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SXT constin among *Vibrio cholerae* isolates from a tertiary care hospital

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***Background & objectives*: The SXT element, also known as 'constin' (conjugable, self transmissible, integrating element) is an integrating conjugative element (ICE) in *Vibrio cholerae* discovered in the chromosome of epidemic *V. cholerae* O139 strain MO10 (SXT^{MO10}) which arose in late 1992 in Chennai, India. SXT related ICEs have become widespread and currently, most if not all Asian *V. cholerae* clinical isolates contain SXT related ICEs. The present study attempts to determine the presence of SXT *Int* gene in *V. cholerae* recovered between 2005 to 2007 in a tertiary care hospital, demonstrate its conjugal nature and also detect co-presence and co-transfer of plasmids in representative isolates.**

***Methods*: This prospective study was done on 116 *V. cholerae* isolates [114- O1 (107 ogawa and 7 inaba) and 2 - Non O1 Non O139 *V. cholerae*] from watery stools between 2005 to 2007 recovered from equal number of patients. PCR was carried out using SXT *Int* specific primers that produced a 592 bp internal fragment of SXT element, and rifampicin resistant strain of *E.coli* K-12 was used as recipient in conjugation experiments to study transfer of SXT, as also co-transfer of resistance to tetracycline, erythromycin, and nalidixic acid. Antibiotic susceptibility was performed against various antibiotics.**

***Results*: Of the 116 isolates, 110 (94.8%) were positive for SXT element by PCR. It was demonstrated in 94.7 per cent of the O1, and 100 per cent of non O1 non O139 *V. cholerae.* All 2005 isolates, 25 per cent of 2006 isolates and 96.6 per cent of 2007 isolates were positive for SXT. Thirty two drug resistance patterns were observed and the 2007 isolates showed resistance to as many as eight antibiotics. The resistance of SXT positive isolates was higher than those of SXT negative and the typical drug resistance pattern corresponding to SXT^{ET} and SXT^{MO10} was shown by only one *V. cholerae* O1 isolate. Successful conjugal transfer of SXT was seen in 31 (88.6%) of the 35 isolates studied without any co-transfer while, presence of plasmids was observed in two of the 31 donor *V. cholerae* studied.**

Interpretation & conclusions*: The demonstration of SXT element and its successful horizontal transfer in *V. cholerae* isolates studied emphasizes the need for its detection to monitor antibiotic resistance and dissemination in *V. cholerae.

Key words Conjugal transfer - constin - drug resistance - SXT ICE - *V. cholerae*

Antibiotic resistance in *Vibrio cholerae* is governed by a complex series of biological, environmental, and behavioural factors. The vast majority of clinically relevant resistance in *V. cholerae* is due to exchange of genetic information among bacterial strains via plasmids and transposons. In addition, mobile gene cassettes and integrons also mediate the rapid and broad dissemination of genetic information across species.

Another type of mobile genetic element; the integrating conjugative elements (ICEs) - self transmissible mobile genetic elements are increasingly being recognized as important mediators of horizontal gene transfer in prokaryotes[1]. These mobile elements have both plasmid and phage-like features. Similar to conjugative plasmids, ICEs transfer via conjugation; but unlike plasmids, these do not replicate autonomously. Similar to many temperate bacteriophages, ICEs integrate into and replicate with the host chromosome[1].

The SXT element, also known as 'constin'[2] (conjugable, self transmissible, integrating element) is one such ICE in *V. cholerae* which was originally discovered in the chromosome of epidemic *V. cholerae* O139 strain MO10 (SXT^{MO10}) that arose in late 1992 in Madras (now Chennai), India[2]. Besides its novel O- antigen, this *V. cholerae* O139 differed from the El Tor O1 *V. cholerae*, it replaced by characteristic resistance to sulphamethoxazole (Su), trimethoprim (TM), chloramphenicol (C) and streptomycin (Sm). In MO10, the genes mediating these resistances were found to be carried by an ICE that was initially called SXT (and later re-named SXTMO10)[1]. After the O139 outbreaks, the re-emerged *V. cholerae* O1 also showed resistance to the same antibioitics as the epidemic *V. cholerae* O139[3] and the corresponding resistance genes were carried by an ICE, originally designated as SXT^{ET}, closely related but not identical to SXT^{MO10} [2]. SXT related ICEs have become widespread in Asian and African countries during the last decade. Currently, most if not all, Asian *V. cholerae* clinical isolates contain SXT related ICEs[1]. SXT element has also been shown to mobilize chromosomal and plasmid DNA with it[4]. Co-transfer of tetracycline resistance determinant *tetA* has been shown with SXT element[5].

In the last decade, the SXT/R391 family of ICEs has been the subject of a growing interest and more than 50 ICEs have been identified and grouped within the SXT/R391 family to date, 30 of these in clinical and environmental *V. cholerae* strains. To date, 15 SXT-related ICEs isolated in India or Bangladesh between 1992 and 2001 have been identified, and six (SXT^{MO10}, ICE*Vch*Ind4, ICE*Vch*Ban5, ICE*Vch*Ban10, ICE*Vch*Ban9, and ICE*Vch*Ind5) of these completely sequenced and annotated[6].

Here, we present the results of a study in which we examined isolates of *V. cholerae* recovered between 2005 to 2007 in a tertiary care hospital in central India, for the presence of SXT *Int* gene by PCR, demonstrated its conjugable nature and also co-presence and co-transfer of plasmids in representative isolates.

Material & Methods

This prospective study was done on 116 *V. cholerae* isolates obtained between 2005 to 2007 from watery stools of equal number of patients attending Kasturba Hospital attached to Mahatma Gandhi Institute of Medical Sciences, Sewagram, Wardha. The study isolates comprised 114- O1 (107 ogawa and 7 inaba) and 2 - Non O1 Non O139 *V. cholerae*. All the isolates were identified using standard procedures[7]. Stool specimens were received in the laboratory within 30 min of collection.

All samples were examined for darting motility by hanging drop and were inoculated in 10 ml of alkaline peptone water (APW, pH 8.6) for enrichment and incubated at 37^0C for 4-6 h. Specimens received in APW were incubated for 4-6 h. After enrichment, motility was rechecked and subculture was done on nonselective media, *i.e.* blood agar and MacConkey agar, and a selective medium thiosulphate citrate bile salt sucrose agar. Specimens were also cultured directly on these media before enrichment.

Serotyping of the strains was done using high titre antisera for *V. cholerae* O1, ogawa and inaba serotypes obtained from Central Research Institute, Kasauli, and O139 antiserum obtained from National Institute of Cholera and Enteric Diseases (NICED), Kolkata.

Antibiotic susceptibility testing: Resistance of *V. cholerae* isolates to ampicillin (in µg) (10), gentamycin (10), tetracycline (30), chloramphenicol (30), cotrimoxazole (25), streptomycin (10), erythromycin (30), trimethoprim (5), nalidixic acid (30) and rifampicin (5) was determined by Kirby Bauers disc diffusion technique using commercial discs (Hi-Media, Mumbai, India) as per CLSI guidelines[8]. ATCC *E.coli* 25922 was used as a control strain.

DNA extraction[9]: *V. cholerae* isolates to be tested for the presence of SXT element were grown on the blood agar plates; 3-4 well isolated colonies of *V. cholerae* were picked up with the help of a sterile loop and inoculated into 100 µl distilled water taken in 1.5 ml Eppendorf tubes. The tubes were then vortexed for a few seconds to make a uniform suspension of the colonies. The suspension was then boiled at 100^0C in a water bath for 30 min in such a way that only the lower part of the Eppendorf tube was immersed in the water using thermacol sheets. After boiling, the tubes were centrifuged at a speed of 5255 *g* for 15 min in a microcentrifuge. Without disturbing the pellette, the supernatant containing the DNA was pippetted out into a new sterile Eppendorf tube. One µl of (25 µg/ ml) RNAse (Bangalore Genei,

Bangalore) solution was added to each of the extracted DNA. The extracted DNA was stored at -20^{0}C till further use (maximum upto 6 months).

PCR was carried out in a thermal cycler (Euroclone from CELBIO design: AR TEFAKT Industries, Italy) by using SXT *Int* specific primers that produced a 592 bp internal fragment of the integrase of the SXT element. The following primer set was used:[10]

INT1-f-5' GCT GGA TAG GTT AAG GGC GG 3'

INT1-b 5' CTC TAT GGG CAC TGT CCA CAT TG 3'.

Briefly, PCR conditions were as follows: 94^{0}C for 5 min, followed by 35 cycles at 94^{0}C for 1 min, 62^{0}C for 1 min and 72^{0}C for 1 min, followed by a final extension at 72^{0}C for 5 min. *V. cholerae O1* SXT *Integrase* gene$^+$ strain (obtained from University of Rome) was used as a positive control and *E.coli* K-12 strain as negative control. After the PCR cycles were over, products were separated by agarose (0.8%) gel electrophoresis in 1X TAE buffer. The separated PCR products were visualized on the UV transilluminator (Bangalore Genei, India).

Transfer of the SXT element[10]: Rifampicin resistant strain of *E. coli* K-12 was used as recipient in conjugation experiments. Total 35 *V. cholerae* isolates were used as donors to study the conjugal transfer of SXT element (33 SXT positive and 2 SXT negative). Of these, 33 were serogroup O1 (27 ogawa and 4 inaba serotype), and 2 were non O1 non O139 *V. cholerae*. All the SXT +ve isolates selected for the conjugal transfer were phenotypically resistant to sulphamethoxazole, trimethoprim and streptomycin in addition to other drugs.

The following drug concentrations (µg/ml) were used for selection in conjugation experiments: sulphamethoxazole (160), trimethoprim (32), rifampicin (50). Conjugation assays were performed by mixing overnight broth cultures of donor and recipient strains in a ratio of 1:1 and centrifuging the mixture at 1892 *g* for 5 min, twice. After mating on non-selective Luria Bertani agar, incubation at 37^{0}C was done for 4-6 h, transconjugants were harvested and appropriate dilutions were spread on Luria Bertani agar plates containing appropriate drug concentrations. The frequency of transfer was expressed as the number of resistant recipient cells per donor cell in the mating mixture at the time of plating (Number of exconjugants /Number of recipients).

The exconjugants of 25 isolates showing resistance to tetracycline and/or erythromycin and/or nalidixic acid were also evaluated for co-transfer of resistance to these antibiotics with SXT element.

Plasmid DNA preparation was obtained by the plasmid DNA minispin-50 Kit (Chromous Biotech, Bangalore) according to manufacturer's instructions. The plasmid DNA was separated by agarose (1.0%) gel electrophoresis in 1X TAE buffer. The separated PCR products were visualized on the UV transilluminator.

Results

Of the 116 isolates studied, 110 *V.cholerae* (94.8%) showed the presence of SXT element by PCR. It was detected in 94.7 per cent of the O1 isolates and 100 per cent of non O1 non O139 *V. cholerae* studied. SXT element was demonstrated in all the isolates of 2005, in 25 per cent (1/4) of 2006 isolates and 96.6 per cent (84/87) of the 2007 isolates. Both the non O1 non O139 *V. cholerae* isolates of 2005 showed the presence of SXT element.

Thirty two drug resistance patterns were observed in the 116 *V.cholerae* isolates included in the study. The 2007 isolates showed maximum drug resistance patterns, with resistance to as many as 8 antibiotics. Resistance was not seen to chloramphenicol in O1 and non O1-O139, to gentamycin in O1 and to erythromycin in non O1-O139. The resistance of SXT positive isolates was higher than those of SXT negative to all the antibiotics studied, except to chloramphenicol and ampicillin where it was reverse and to gentamycin where it was the same. Among the SXT +ve *V. cholerae* isolates, the typical drug resistance pattern corresponding to SXT^{ET} and SXT^{MO10} was shown by only one *V. cholerae* O1 isolate belonging to the year 2005. *V. cholerae* O1 isolates (n=101) and both the SXT +ve non O1 non O139 isolates showed resistance to co-trimoxazole, trimethoprim and streptomycin. The remaining isolates (13 - *V. cholerae* O1) showed resistance to the antibiotics coded for by the SXT^{ET} / SXT^{MO10} in various combinations.

Of the total 35 SXT +ve isolates tested for the conjugal transfer of SXT element, transfer was successful in 31 (88.6%). All the exconjugants showed resistance to co-trimoxazole, trimethoprim and streptomycin. The frequency of transfer ranged from 1 X10^{-5} exconjugants/recipient to 7 X 10^{-6} exconjugants/recipient. No co-transfer of resistance to tetracycline, erythromycin or nalidixic acid was observed with SXT element, either alone or in combination in the 35 SXT positive isolates studied.

Among the 31SXT +ve isolates showing conjugal transfer of the element, presence of plasmids was observed in two donor *V. cholerae* and also in their exconjugant *E. coli-K12*.

Discussion

Integrative and conjugative elements (ICEs) form a large class of mobile genetic elements that are able to encode many properties including drug resistance. The SXT element is an ICE that contributes to horizontal transmission and rearrangement of resistance genes in *V. cholerae*[10]. PCR using *int1-f* and *int1-b* primer pair has been successfully used in the present study to produce a 592 bp internal fragment of the *integrase* of the SXT element in both O1 and non O1 non O139. Using this primer sets, Dalsgaard *et al*[5] demonstrated the presence of SXT element in all the 20 *V. cholerae* isolates studied, while Ceccarelli *et al*[10] demonstrated it in 6 *V. cholerae* O1out of 13 (12- O1,1- non O1) received from different regions of Angola.

Ramachandran *et al*[11] while using a different primer set were able to demonstrate SXT element in 97.2 per cent of *V. cholerae* O1, 59.7 per cent of *V. cholerae* O139 and 12.5 per cent of non O1 non O139 isolates from different parts of India. This study also included 16 SXT positive O139 isolates of 1997 from Sevagram, Wardha. Another study[12] using the similar primers demonstrated the 1035 bp SXT amplicon in all the 10 aquatic *V. cholerae* O139 studied, and Okoh *et al*[13] in vibrios isolated from waste water effluent. A study from Kolkata demonstrated its presence in 38 out of 58 *V.cholerae* O139 isolates studied[14].

In our study, SXT element was found in 96.6 per cent of isolates in 2007 as also observed by others[5,10]. SXT element was not only demonstrated in ogawa serotype but also in all inaba isolates, a finding which correlated with that reported earlier[11,15].

In the present study, only one *V. cholerae* O1 SXT +ve isolate showed the typical resistance pattern (Su, Tm, Sm, C) as shown by SXT^{MO10} and SXT^{ET} *V. cholerae.* None of the other Indian studies reviewed have demonstrated the presence of this typical resistance pattern in their SXT positive isolates. The most common resistance pattern observed in our SXT +ve isolates with respect to the drugs coded by the SXT element was SuTSmTm (31 *V. cholerae* O1). Similar findings have been reported in 51 *V. cholerae* O1 isolates by Ramchandran *et al*[11]. Mohpatra *et al*[15] have reported this pattern both in ogawa and inaba serotypes as has also been observed by us. In the present study, one of the 2007 SXT +ve *V. cholerae* O1 isolate lacked resistance to all of the drugs coded for by the SXT^{MO10} and SXT^{ET} (Su, Tm, Sm, C). The SXT element has been shown to delete antibiotic-resistance genes to improve its evolutionary fitness, possibly depending on the use of the corresponding antibiotics in human or animal disease treatment or prophylaxis[16].

In the present study successful transfer of the SXT element to *E. coli* K-12 was demonstrated in 31 (O1 as well as non O1 non O139) of the 35 isolates studied. Failure to generate the intermediate circular form of SXT is responsible for its unsuccessful transfer[2] as has been observed by others[10]. The frequency of transfer observed was as high as $7x\ 10^{-6}$ in O1 serogroup (range- $1x10^{-5}$ to $7x\ 10^{-6}$) and $3\ x\ 10^{-6}$ within non O1 non O139 *V. cholerae.* The frequency of transfer has been shown to be $2\ X\ 10^{-7}$ to SXT^{S} El Tor *V. cholerae* and $3\ x\ 10^{-9}$ to SXT^{S} classical *V. cholerae*[17].

Hothhut *et al*[4] have demonstrated that the gene transfer capacity of the SXT element goes beyond its self-transfer. They demonstrated that in *E. coli* K-12 the SXT element also mobilized certain plasmids in *trans* and transferred chromosomal DNA in a directional fashion in *cis.* Another group[10] has demonstrated the co-presence of SXT element with plasmids and class 1 integrons in clinical as well as environmental *V. cholerae* isolates, whereas Amita *et al*[14] did not find plasmid in any of their isolates. Thungapatra[18] demonstrated SXT ICE in 12 of the 43 non O1 non O139 isolates harboring plasmids. In our study, we also tested for the presence of plasmids in 31 SXT positive *V. cholerae* isolates and further studied its co-transfer with SXT element. Only 2 of these 31 isolates studied showed the presence of plasmids. The plasmids were also demonstrated in the exconjugants obtained after conjugation experiments using 2 strains as donors. However, we did not look for the SXT element in these exconjugants, hence are not able to conclude that the resistance to antibiotics coded for by the SXT element observed in the exconjugants is due to SXT element or the plasmid.

In a study conducted in Mozambique and South Africa[5], tetracycline resistant determinants (*tet*A) were found to be transferable in conjugation experiments with the SXT element. In another study conducted at Dhaka[19] transfer of tetracycline resistance was always linked with the transfer of resistance to streptomycin, SXT and erythromycin but not with resistance to nalidixic acid. In the present study, we tested the co-transfer of erythromycin, tetracycline and nalidixic acid with the SXT element. For this the exconjugants

obtained after conjugation experiments were tested for resistance to tetracycline, erythromycin and nalidixic acid by disc diffusion method in addition to the drugs coded by the SXT element. No co-transfer of these drugs was observed with the SXT element either alone or in combinations.

Broad dissemination of the SXT element in *V. cholerae* O1 and O139 strains not only in Indian subcontinent but also other regions of the world suggests that this element confers some selective advantage to *V. cholerae*. It has been shown that the SXT element does not encode an intestinal colonizing factor[17], but it might be playing some other role in the virulence of *V. cholerae* or in the environmental ecology.

In conclusion, the demonstration of SXT element and its successful horizontal transfer in the studied isolates, emphasizes the need for its detection to monitor antibiotic resistance and dissemination in *V. cholerae.*

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References

1. Burrus V, Marrero J, Waldor M. The current ICE age: Biology and evolution of SXT-related integrating conjugative elements. *Plasmid* 2006; *55* : 173-83.
2. Hochhut B, Waldor M. Site specific integration of the conjugal *Vibrio cholerae* SXT element into *prfC*. *Mol Microbiol* 1999; *32* : 99-110.
3. Yamamoto T, Nair GB, Albert MJ, Parodi CC, Takeda Y. Survey of *in vitro* susceptibilities of *Vibrio cholerae* O1 and O139 to antimicrobial agents. *Antimicrob Agents Chemother* 1995; *39* : 241-4.
4. Hochhut B, Marrero J, Waldor M. Mobilization of plasmids and chromosomal DNA mediated by the SXT element, a constin found in *Vibrio cholerae* O139. *J Bacteriol* 2000; *182* : 2043-7.
5. Dalsgaard A, Forslund A, Sandvang D, Arntzen L, Keddy K. *Vibrio cholerae* O1 outbreak in Mozambique and South Africa in 1998 are multiple-drug resistant, contain the SXT element and the *aadA2* gene located on class I integrons. *J Antimicrob Chemother* 2001; *48* : 827-38.
6. Ceccarelli D, Spagnoletti M, Bacciu D, danin-Poleg Y, Mendiratta DK, Koshi Y, *et al*. ICE*Vch*Ind5 is prevalent in epidemic *Vibrio cholerae* O1 El Tor strains isolated in India. *Int J Med Microbiol* 2011; *301* : 318-24.
7. Collee JG. *Mackie & Mccartney practical medical microbiology*. New York: Churchill Livingstone; 1996.
8. Clinical Laboratory Standard Institute (CLSI). *Performance standards for antimicrobial testing*. 15th informational supplement (M100-S15) vol.25, No.1. Wayne, PA: CLSI; 2005.
9. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, *et al*. Current protocols in molecular biology. New York: Greene Publishing Associates and Wiley Interscience; 1990.
10. Ceccarelli D, Salvia AM, Sami J, Cappuccinelli P, Colombo MM. New cluster of plasmid-located class 1 integrons in *Vibrio cholerae* O1 and a dfrA15 cassette-containing integron in *Vibrio parahaemolyticus* isolated in Angola. *Antimicrob Agents Chemother* 2006; *50* : 2493-9.
11. Ramachandran D, Bhanumathi R, Singh DV. Multiplex PCR for detection of antibiotic resistance genes and the SXT element: application in the characterization of *Vibrio cholerae. J Med Microbiol* 2007; *56* : 346-51.
12. Bhanumathi R, Sabeena F, Isac SR, Shukla BN, Singh DV. Molecular characterization of *Vibrio cholerae* O139 Bengal isolated from water and the aquatic plant *Eichhornia crassipes* in the River Ganga, Varanasi, India. *Appl Environ Microbiol* 2009; *69* : 2389-94.
13. Okoh AI, Igbinosa EO. Antibiotic susceptibility profiles of some *Vibrio* strains isolated from wastewater final effluents in a rural community of the Eastern Cape Province of South Africa. *BMC Microbiol* 2010; *10* : 143.
14. Amita, Roy Chowdhary S, Thungapatra M, Ramamurthy T, Nair GB, Ghosh A. Class I integrons and SXT elements in El Tor strains isolated before and after 1992 *Vibrio cholerae* O139 outbreak, Calcutta, India. *Emerg Infect Dis* 2003; *9* : 500-2.
15. Mohapatra SS, Ramachandran D, Mantri CK, Singh DV. Characterization of the genetic background of *Vibrio cholerae* O1 biotype El Tor serotype Inaba strains isolated in Trivandrum, southern India. *J Med Microbiol* 2007; *56* : 260-5.
16. Hochhut B, Lotfi Y, Mazel D, Faruque SM, Woodgate R, Waldor M. Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. *Antimicrob Agents Chemother* 2001; *45* : 2991-3000.
17. Waldor MK, Tschape H, Mekalanos JJ. A new type of conjugative transposon encodes resistance to sulphamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. *J Bacteriol* 1996; *178* : 4157-65.
18. Thungapatra M. Occurrence of antibiotic resistant Gene Cassettes *aac(6')-Ib, dfrA5, dfrA12, ereA2* in class I integrons in Non O1 Non O139 *Vibrio cholerae* strains in India. *Antimicrob Agents Chemother* 2002; *46* : 2948-55.
19. Faruque SM, Islam MJ, Ahmad QS, Biswas K, Faruque ASG, Nair GB, *et al*. An improved technique for isolation of environmental *Vibrio cholerae* with epidemic potential: Monitoring the emergence of a multiple- antibiotic -resistant epidemic strain in Bangladesh. *J Infect Dis* 2006; *193* : 1029-36.

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