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# Genetic Analysis of CTX Prophage and Antibiotic Resistance Determinants in *Vibrio cholerae* O1 Belonging to the Atypical El Tor Biotype from Kelantan, Malaysia

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## 1. Introduction

Epidemic and global pandemic cholera have claimed millions of lives since the first pandemic in 1817 and it continues to exact a huge annual toll, with endemics established in approximately 50 countries worldwide (Ryan, 2011). More than 200 serogroups have been reported to date (Safa et al., 2009), but only two serogroups of *Vibrio cholerae*, the O1 and O139 serogroups, are known to have the potential for unleashing epidemic and pandemic cholera. The O1 serogroup of *V. cholerae* can be further divided into the classical or El Tor biotypes based on a number of phenotypic and genotypic characteristics (Sack et al., 2004). Seven pandemics of cholera have been recorded to date, where the first six pandemics were associated with *V. cholerae* serogroup O1 of the classical biotype, whereas the current ongoing seventh pandemic (1961 until present day) is caused by *V. cholerae* serogroup O1 of the El Tor biotype (Faruque et al., 1998). In 1992, the emergence and rapid spread of *V. cholerae* serogroup O139 from the Indian subcontinent to neighbouring countries was viewed as a possible threat that might initiate an eighth cholera pandemic. At the height of the O139 outbreak, the Indian subcontinent saw a dramatic displacement of the *V. cholerae* O1 El Tor as the dominant strain. However, rather than being driven to gradual extinction like the classical biotype, an unprecedented turn of events in 1994 saw *V. cholerae* O1 El Tor regain its predominance over the O139 serogroup and both serogroups continue to cause disease on the Indian subcontinent (Faruque et al., 2003).

The current seventh pandemic rein has reached its half a century mark, but several variants of the *V. cholerae* O1 El Tor biotype emerged cryptically during the late 1990s. These variants were untypable according to the conventional biotyping classification, because they possessed traits of both classical and El Tor biotypes (Nair et al., 2002; Ansaruzzaman et al., 2004). Given this dilemma, Safa et al. (2009) proposed the designation 'atypical El Tor' as an umbrella term to encompass all variants of the El Tor biotype. One such atypical El Tor was collectively known as the Matlab variants isolated from hospitalized patients in Matlab, Bangladesh between 1991 and 1994. Chronologically, the Matlab variants were the first to be characterized as having attributes of both the classical and El Tor biotypes, which meant

that they could not be differentiated into a specific biotype based on their phenotypic traits (Nair et al., 2002). In 2004, a second variant, designated as the Mozambique variant, was found to have typical El Tor phenotypic traits but it genetically harboured a tandem repeat of the classical CTX prophage on the small chromosome (Ansaruzzaman et al., 2004). The third, and perhaps the most significant atypical El Tor, was the altered El Tor that is uniquely recognized as carrying the classical cholera toxin while retaining almost all aspects of the prototypic seventh pandemic El Tor strain.

This altered El Tor was initially reported in Bangladesh and growing evidence suggests the wide spread of this variant around the world in recent years (Nguyen et al., 2009; Okada et al., 2010; Morita et al., 2010; Sithivong et al., 2010; Ceccarelli et al., 2011). The altered El Tor was reported to have fully displaced prototypic seventh pandemic El Tor strains in several countries, including India and Bangladesh (Nair et al., 2006; Raychoudhuri et al., 2009). Two different conjectures have been proposed for the emergence and global transmission of atypical El Tor (Alam et al., 2010). The emergence of altered El Tor was postulated to be the result of either clonal expansion of a single ancestral El Tor which had acquired the classical *ctxB* gene in a cholera endemic region or a multiclonal event occurring independently in each region from co-existing El Tor and classical strains. Transnational transmission of altered El Tor was exemplified by the recent 2010 Haiti outbreak which was thought to be introduced by human activity from South Asian countries (Chin et al., 2011) and subsequently spread to United States, Canada and Dominican Republic via importation by travellers from Haiti (CDC, 2010; Gilmour et al., 2011).

The emergence of atypical El Tor marks a significant event in the evolution of *V. cholerae* and the epidemiology of cholera. The 2009 cholera outbreak strain from Kelantan state on the east coast of peninsular Malaysia was characterized as belonging to the altered El Tor biotype and it carried the classical cholera toxin (*ctxB*) gene (Ang et al., 2010). The present study further investigated genetic aspects of the Kelantan altered El Tor strain using multiple PCR analysis to elucidate the structure of the CTX prophage and to detect the presence of class I integron and SXT element antibiotic determinants.

## 2. Materials and methods

### 2.1 *V. cholerae* strains

A total of 20 *V. cholerae* isolates belonging to serogroup O1 of the altered El Tor biotype were collected during the 2009 cholera outbreak in Kelantan, Malaysia as described earlier (Ang et al., 2010). All the *V. cholerae* isolates were revived from glycerol stock and identification was performed using standard biochemical methods (Kay et al., 1994). Serotyping was conducted using slide agglutination tests with polyvalent O1 and monospecific Ogawa and Inaba antisera (Denka Seikan, Japan). All isolates were routinely grown on Luria-Bertani (LB) agar throughout the study.

### 2.2 Genomic DNA preparation

The genomic DNA template for genetic analysis was purified using a NucleoSpin Tissue kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. The purity and concentration of purified genomic DNA was determined using a Biophotometer (Eppendorf, Germany).

### 2.3 *rstR* typing

The type of *rstR* gene in each isolate was determined using a set of allele-specific forward primers (*rstR*<sup>El Tor</sup>, *rstR*<sup>Classical</sup>, *rstR*<sup>Calcutta</sup> and *rstR*<sup>Environment</sup>) and a common reverse primer, *rstRR* (Nusrin et al., 2004; Bhattacharya et al., 2006). A list of all primers used in this study is presented in Table 1.

Primer	Nucleotide sequence (5' to 3')	References
<i>rstR</i> <sup>Classical</sup>	CTTCTCATCAGCAAAGCCTCCATC	Bhattacharya et al., 2006
<i>rstR</i> <sup>El Tor</sup>	GCACCATGATTTAAGATGCTC	Bhattacharya et al., 2006
<i>rstR</i> <sup>Calcutta</sup>	CTGTAAATCTCTTCAATCCTAGG	Bhattacharya et al., 2006
<i>rstR</i> <sup>Environment</sup>	GTAAACGCTTCAAGCCTG	Nusrin et al., 2004
<i>rstRR</i>	TCGAGTTGTAATTCATCAAGAGTG	Bhattacharya et al., 2006
Ch1F	GACCACTCAGGCCGCTGAAAT	Nguyen et al., 2009
Ch1R	CCGCGCTCAAGTGGTTATCGG	Nguyen et al., 2009
Ch2F	AACAACAGGTTGCAAGAGAGCATT	Nguyen et al., 2009
Ch2R	TATTGCTTTTTTAATGGCCGTT	Nguyen et al., 2009
rstAR	CCGTGAAAGTCATCAACG	Nguyen et al., 2009
rstCF	GATGTTTACGATAGCCTAGAAGACTT	Nguyen et al., 2009
rstCR	TACAGTGATGGCTCAGTCAATGC	Nguyen et al., 2009
ctxBF	AGATATTTTCGTATACAGAATCTCTAG	Nguyen et al., 2009
cepR	AAACAGCAAGAAAACCCCGAGT	Nguyen et al., 2009
rstCF4	AAATCCGCAACTCAAGGCATTGA	Nguyen et al., 2009
rstCR4	TAAGCGCCTGAACGCAGATATAAAG	Nguyen et al., 2009
<i>rtxC-F</i>	CGACGAAGATCATTGACGAC	Chow et al., 2001
<i>rtxC-R</i>	CATCGTCGTTATGTGGTTGC	Chow et al., 2001
inDS-F	CGGAATGGCCGAGCAGAT C	Dalsgaard et al., 2001
inDS-B	CAAGGTTCTGGACCAGTTGCG	Dalsgaard et al., 2001
qacEΔ1-F	ATCGCAATAGTTGGCGAAGT	Dalsgaard et al., 2001
su1-B	GCAAGGCGGAAACCCGCGCGG	Dalsgaard et al., 2001
in-F	GGCATCCAAGCAGCAAGC	Dalsgaard et al., 2001
in-B	AAGCAGACT TGACCTGAT	Dalsgaard et al., 2001
aadA-B	ATTGCCAGTCGGCAGCG	Dalsgaard et al., 2001
INT1	GCTGGATAGGTTAAGGGCAG	Hochhnut et al., 2001
INT2	CTCTATGGGCACIGTCCACATTG	Hochhnut et al., 2001
Sul2-F	AGG GGG CAG ATG TGATCGAC	Hochhnut et al., 2001
Sul2-B	TGTGCGGATGAAGTCAGCTCC	Hochhnut et al., 2001
FLOR-F	TTATCTCCCTGTCGTTCCAGCG	Hochhnut et al., 2001
FLOR-2	CCTATG AGCACACGGGGAGC	Iwanaga et al., 2004
strB-F	GGCACCCATAAGCGTACGCC	Iwanaga et al., 2004
strB-R	TGCCGAGCACGGCGACTACC	Iwanaga et al., 2004
DFR1-F	CGAAGAATGGAGTTATCGGG	Iwanaga et al., 2004
DFR1-B	TGCTGGGGATTTCAGGAAAG	Iwanaga et al., 2004

Table 1. Primers used in this study

## 2.4 Genetic analysis of CTX prophage array

Genetic analysis of CTX prophage array was performed using several combinations of primer pairs, as described by Nguyen et al. (2009). The presence of a RS1 element was determined using the primer pair *rstCF/rstCR*. Investigations of the arrays for RS1 and CTX prophage were performed using two primer pairs: *ctxBF/rstCR* for the CTX prophage-RS1 array and *rstCF4/rstAR* for the RS1-CTX prophage array. The presence of tandem repeats of the RS1 element or CTX prophage was determined using the primer pair *rstCF4/rstCR4* and *ctxBF/cepR*, respectively. The chromosomal localization of RS1 and CTX prophage was confirmed using the primer pairs *Ch1F/rstRR* and *ctxBF/Ch1R* for the large chromosome and *Ch2F/Ch2R* for the small chromosome.

## 2.5 *rtxC* PCR

Detection of the *rtxC* gene was performed using the primer pair *rtxC-F/rtxC-R* (Chow et al., 2001).

## 2.6 PCR detection of class I integrons

Detection of class I integrons was performed using a set of primers described by Dalsgaard et al. (2001). Briefly, the primer pairs *inDS-F/inDS-B* and *qacEΔ1-F/su1-B* were used for the amplification of the 5'-CS and 3'-CS of the class I integron. The primer pair *in-F/in-B* was used to amplify gene cassettes inserted in the integron, while the primer pair *in-F/aadA-B* was used for the amplification of the gene cassette *aad1A* that encodes streptomycin resistance.

## 2.7 PCR detection of SXT constins

The isolates were screened for the presence of SXT constins (large conjugative elements) using the primer pair *INT1/INT2*. Presence of the antibiotic resistance genes *floR* that encodes resistance to chloramphenicol, *sulII* for resistance to sulfamethoxazole, *strB* for resistance to streptomycin, and *dfrA1* for resistance to trimethoprim, were determined using the primer pairs *FLOR-F/FLOR-2*, *Sul2-F/Sul2-B*, *strB-F/strB-R*, and *DFR1-F/DFR1-B*, respectively (Hochhut et al., 2001).

## 2.8 Sequencing of tandem repeats of RS1 elements and upstream regions of the CTX prophage

The DNA sequence spanning tandem repeats of RS1 elements and upstream regions of the CTX prophage (~8.5 kb) was generated with the primer pairs *Ch1F/rstCR*, *rstCF4/rstCR4*, and *rstCF/cepR*. Amplicons from each PCR reaction were cloned into the pCR4-TOPO vector (Invitrogen, CA) and sequenced by First Base Laboratories Sdn. Bhd. (Malaysia). The sequencing data was assembled and complete nucleotide sequences of the RS1-RS1-CTX prophage arrays for isolates 03/09-KB and 27/09-KB were deposited in GenBank under the accession numbers JN545744 and JN545745.

## 2.9 Sequencing of PCR amplicons

The nucleotide sequences of the PCR amplicons generated by each of the primer pairs *Ch2F/Ch2R*, *INT1/INT2*, *Sul2-F/Sul2-B*, *strB-F/strB-R*, *DFR1-F/DFR1-B*, and *rtxC-F/rtxC-*

*R* were confirmed by sequencing reactions. Prior to being sequenced, all amplicons from positive PCR reactions were purified using Wizard SV Gel and PCR Clean-up System (Promega, Australia), according to the manufacturer's instructions. The complete nucleotide sequences of *Sui1* gene amplified using the primer pair Ch2F/Ch2R for isolates 03/09-KB and 27/09-KB were assigned the GenBank accession numbers JN545747 and JN545748. The partial nucleotide sequences of *rtxC*, SXT element, *SullI*, *strB*, and *dfrA1* for isolate 03/09-KB were deposited under accession numbers JN545752, JN545751, JN545754, JN545753, and JN545750, respectively.

### 3. Results

All the revived isolates were identified as *V. cholerae* biotype El Tor using standard biochemical tests and slide agglutination tests showed they belonged to serogroup O1 of the Ogawa serotype.

#### 3.1 *rstR* typing

The *rstR* typing by PCR amplification of the 501 bp amplicon using the primer pair *rstR<sup>El Tor</sup>*/*rstRR* showed that all isolates possessed only the El Tor type *rstR* (Fig. 1). No amplicon was produced for other allele-specific primers among all isolates analyzed, which indicated the absence of classical, Calcutta, and environmental type *rstR*.

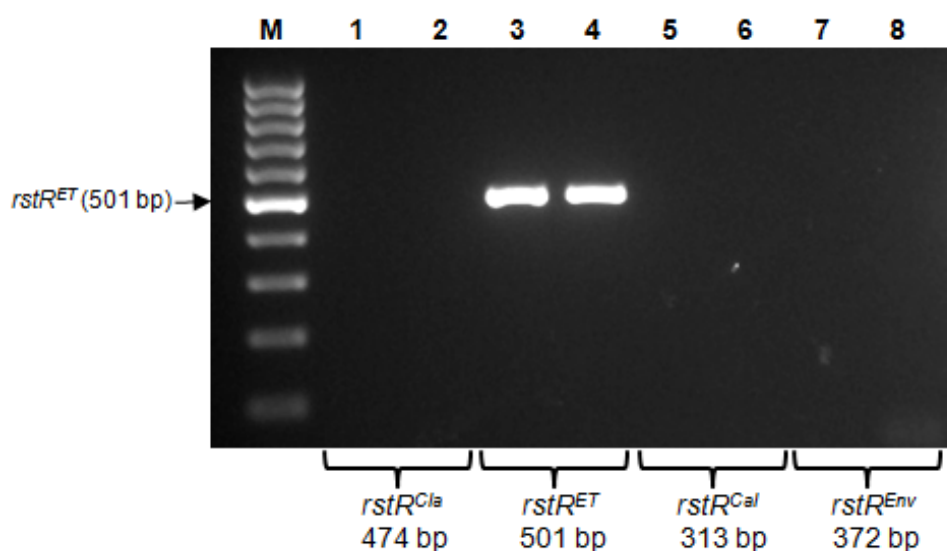


Fig. 1. Agarose gel electrophoresis products of *rstR* typing. The expected product size and types of *rstR* targeted by specific primer pairs are indicated below the gel. Lane M: 100 bp DNA ladder; lanes 1, 3, 5, and 7: representative isolate 03/09-KB; lanes 2, 4, 6, and 8: representative isolate 27/09-KB. *rstR<sup>Cla</sup>*: classical type *rstR*; *rstR<sup>ET</sup>*: El Tor type *rstR*; *rstR<sup>Cal</sup>*: Calcutta type *rstR*; *rstR<sup>Env</sup>*: environmental type *rstR*.

#### 3.2 Genetic analysis of the CTX prophage array

The presence of RS1 element was confirmed using the primer pair *rstCF*/*rstCR* to amplify a 197 bp region of *rstC* gene from all the isolates (Fig. 2a). The CTX prophage and RS1 element in each isolate was found to be arranged in the form of a RS1-CTX prophage array, as shown

by the positive amplification of a 1551 bp amplicon with the primer pair *rstCF4/rstAR* (Fig. 2b). However, no additional RS1 element was found downstream of the CTX prophage because no amplicon was generated by the primer pair *ctxBF/rstCR*. The primer pair *rstCF4/rstCR4* indicated the tandem arrangement of the RS1 element through the amplification of a 2629 bp amplicon, whereas no amplicon was produced for the primer pair *ctxBF/cepR* which indicated the presence of only a single CTX prophage. The location of the RS1-RS1-CTX prophage array on the large chromosome was verified using the primer pairs *Ch1F/rstRR* and *ctxBF/Ch1R* which amplify fragments corresponding to the upstream and downstream regions of the RS1-RS1-CTX prophage array found on the large chromosome. The absence of an RS1 element or CTX prophage on the small chromosome was confirmed through the amplification of a 910 bp amplicon using the primer pair *Ch2F/Ch2R*.

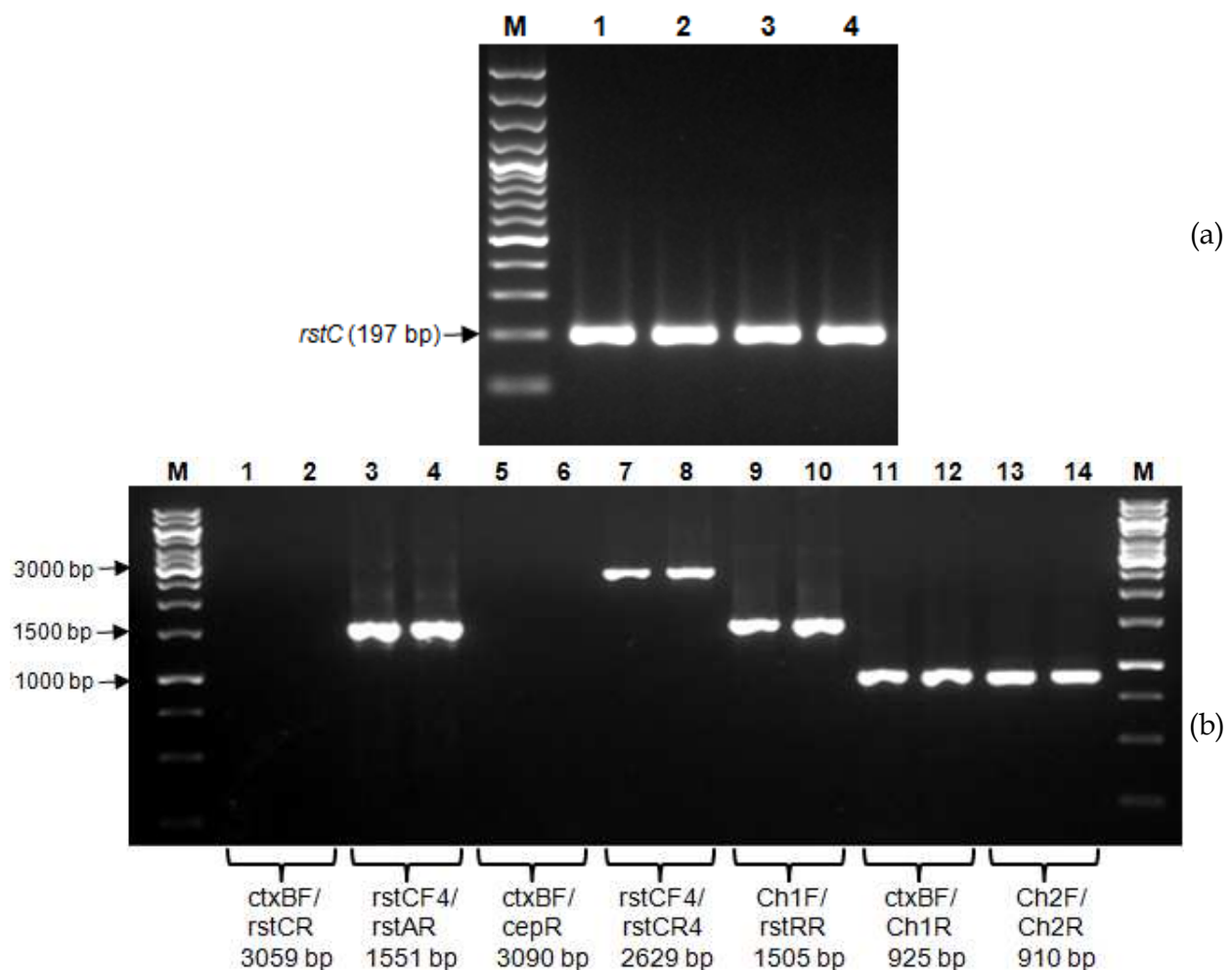


Fig. 2. (a) Agarose gel electrophoresis products in the detection of RS1 elements from representative isolates. Lane M: 100 bp Plus DNA ladder; lane 1: isolate 03/09-KB; lane 2: isolate 11/09-KB; lane 3: isolate 27/09-KB; lane 4: isolate 29/09-KB. (b) Agarose gel electrophoresis products from the analysis of the CTX prophage array. The combinations of different primer pairs and their expected product sizes are indicated below the gel. Lane M: 1 kb DNA ladder; lanes 1, 3, 5, 7, 9, 11, and 13: representative isolate 03/09-KB; lanes 2, 4, 6, 8, 10, 12, and 14: representative isolate 27/09-KB.

### 3.3 *rtxC* PCR

PCR analysis of *rtxC* showed that all the isolates yielded a 263 bp amplicon of the *rtxC* gene (Fig. 3).

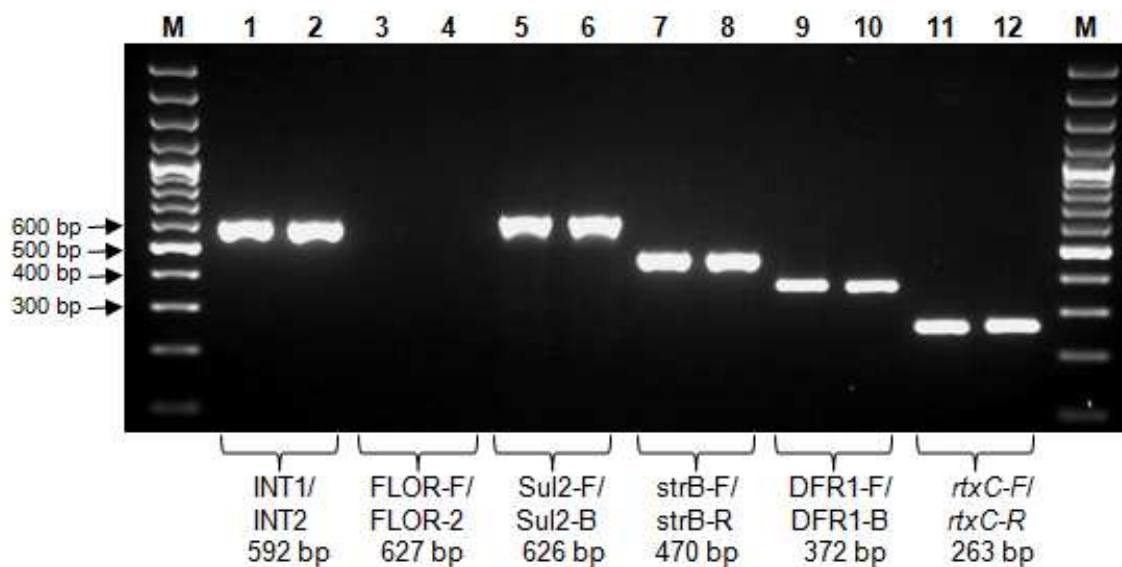


Fig. 3. Agarose gel electrophoresis products from the detection of the *rtxC* gene and the analysis of the SXT constin. The combinations of different primer pairs and their expected product sizes are indicated below the gel. Lane M: 100 bp Plus DNA ladder; lanes 1, 3, 5, 7, 9, and 11: representative isolate 03/09-KB; lanes 2, 4, 6, 8, 10, and 12: representative isolate 27/09-KB.

### 3.4 PCR detection of class I integrons

None of the isolates were positive for class I integrons, because no PCR amplicons were obtained with all the primer pairs tested.

### 3.5 PCR detection of SXT constins

All the isolates were shown to be positive for SXT element by the amplification of a 592 bp amplicon with the primer pair INT1/INT2 (Fig. 3). These isolates also had positive PCR results for the *SulIII*, *strB*, and *dfrA1* genes, because 626 bp, 470 bp, and 372 bp amplicons were generated for the respective genes. None of the isolates were positive for *floR* genes.

## 4. Discussion

In November 2009, Kelantan was struck by a cholera outbreak that marked the re-emergence of this secretory diarrheal disease in the eastern state after years of absence. The aetiological agent was later found to be *V. cholerae* O1 of the altered El Tor biotype and this discovery indicated the first reported appearance of the atypical El Tor strain on Malaysian soil (Ang et al., 2010). Various genetic studies have been undertaken to gain insights into the evolution of this predominant atypical strain and research into the altered El Tor strain has primarily been directed towards the CTX prophage encoding the classical cholera toxin gene (Nguyen et al., 2009; Lee et al., 2009).



The CTX prophage found in the genome of pathogenic *V. cholerae* strains is actually an integrated form of a lysogenic filamentous bacteriophage known as the CTX phage (CTX $\Phi$ ). CTX $\Phi$  is approximately 7 kb in length and it is composed of a 4.6 kb core region with a 2.4 kb RS2 region (Waldor & Mekalanos, 1996). The core region contains genes that encode for proteins involved in phage morphogenesis, specifically the core-encoded pilin (*cep*), pIII<sup>CTX</sup> (previously known as *orfU*), accessory cholera enterotoxin (*ace*), and zonula occludens (*zot*) genes. This core region also contains genes encoding for cholera toxin, so the acquisition of CTX $\Phi$  is viewed as virulence acquisition by a host cell. The RS2 region complements the core, because it contains genes that enable the replication (*rstA*), integration (*rstB*), and regulation (*rstR*) of CTX $\Phi$ . The RS1 element is another RS2-like element that is frequently found adjacent to the CTX prophage. The RS1 element is a 2.7 kb satellite phage that only differs from RS2 by an additional gene designated *rstC*, which encodes for a novel antirepressor to the RstR protein (Waldor et al., 1997; Heilpern & Waldor, 2003). The RS1 element provides a dual function by promoting the transcription of phage genes via an interaction between RstC and RstR, as well as enabling the replication of an adjacent CTX prophage to produce infective phage particles (Davis et al., 2002).

The *rstR* regulatory gene sequence found in the RS2 region also determined the type of CTX prophage carried by a *V. cholerae* strain. Three types of CTX prophage has been established to date, i.e., the classical CTX prophage and El Tor CTX prophage that were first detected in the *V. cholerae* serogroup O1 of the respective biotypes, and the Calcutta CTX prophage from the epidemic-causing serogroup O139 (Kimsey et al., 1998). A fourth type, the Mozambique CTX prophage, was proposed by Choi et al. (2010) and described based on the inclusion of other genetic features of the CTX prophage, including intergenic sequences and the *rstA* gene, although the CTX prophage contained a classical *rstR* gene. In the present study, the CTX prophage from the 2009 Kelantan cholera outbreak strain was found to be regulated by an El Tor type *rstR* repressor gene, but it carried a classical type *ctxB* gene. A CTX prophage with this combination of El Tor type *rstR* and classical cholera toxin gene was also designated as a hybrid CTX prophage by Grim et al. (2010). Further genetic analysis of the CTX prophage structure revealed that all isolates harboured a RS1-RS1-CTX prophage array, which was integrated on the large chromosome. As found in the prototypic seventh pandemic El Tor strains, no RS1 element or CTX prophage was integrated on the small chromosome. The RS1-RS1-CTX prophage array of the Kelantan variant represents a novel arrangement for these genetic elements among atypical El Tor strains. To the best of our knowledge, no RS1-RS1-CTX prophage array with an El Tor type *rstR* on the large chromosome has been demonstrated or reported elsewhere among the altered El Tor biotypes.

In 2009, Nguyen et al. were the first to characterize and report the CTX prophage array of altered El Tor strains isolated during cholera outbreaks that occurred in Vietnam between 2007 and 2008. All the Vietnamese isolates were found to contain the RS1-CTX prophage array with an El Tor type *rstR* on the large chromosome. Similarly, 400 *V. cholerae* isolates obtained between 2003 and 2007 from Kolkata, India were also characterized as having the RS1-CTX prophage array (Nguyen et al., 2009). Recently, the same RS1-CTX prophage array was identified in the altered El Tor isolates from Angola, Africa in 2006 (Ceccarelli et al., 2011) and from Hyderabad, India in 2009 (Goel et al., 2011). A study conducted by Goel et al. (2011) found that one of the altered El Tor isolates (VCH35) from Hyderabad, India harboured a tandem repeat of the CTX prophage in the small chromosome in addition to a RS1-CTX

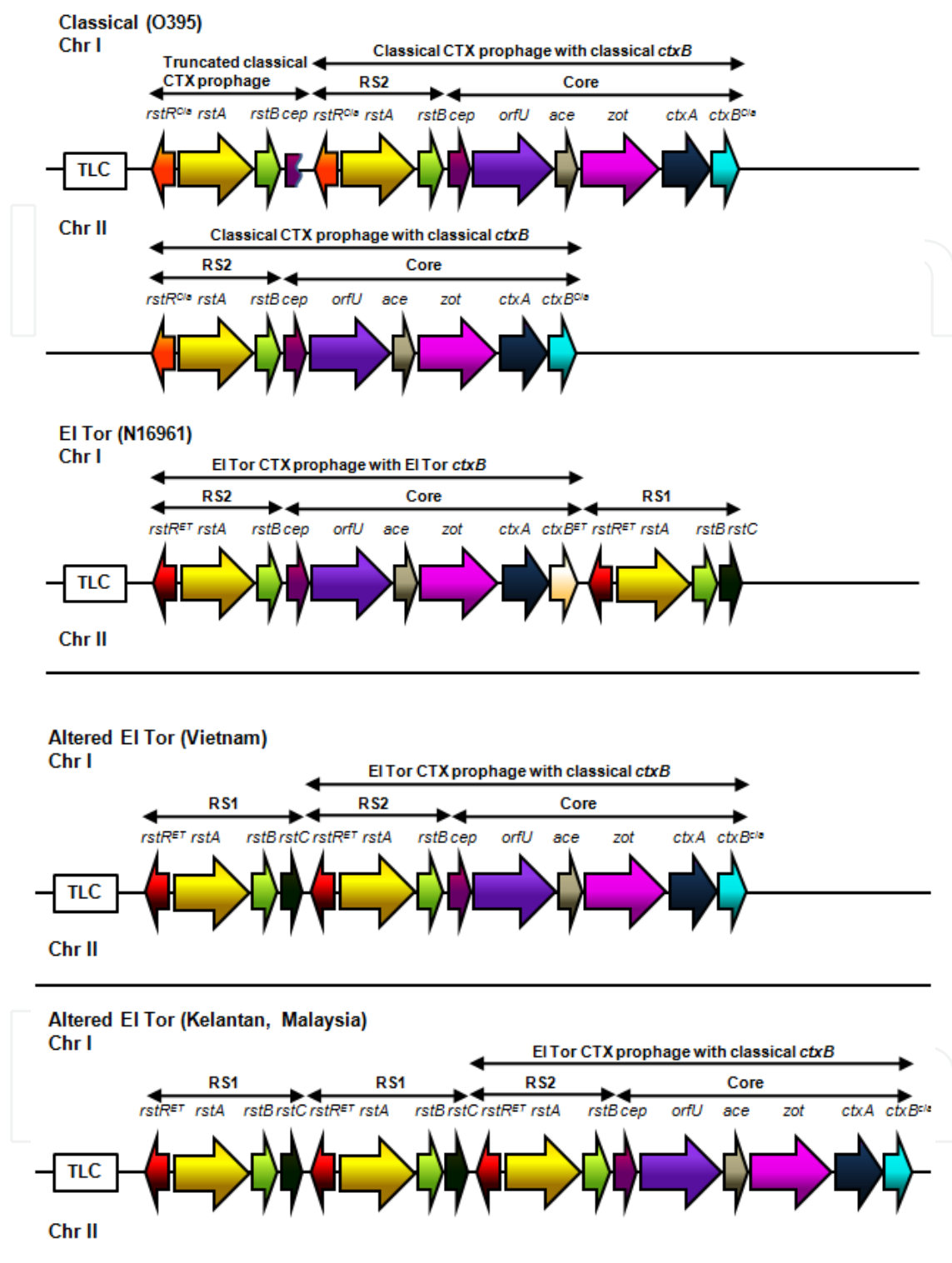


Fig. 4. Genetic map comparison of the CTX prophage arrays found in the classical reference strain (O395), EI Tor reference strain (N16961), Vietnam altered EI Tor, and the Kelantan altered EI Tor characterized in this study. The transcription direction of each gene is indicated by arrows and each gene is shaded in different colours. Chr I: chromosome I; Chr II: chromosome II; *rstR<sup>ET</sup>*: EI Tor type *rstR*; *rstR<sup>Cl<sup>a</sup></sup>*: classical type *rstR*; *ctxB<sup>ET</sup>*: EI Tor type *ctxB*; *ctxB<sup>Cl<sup>a</sup></sup>*: classical type *ctxB*, TLC: toxin-linked cryptic. The map is not drawn to scale.

prophage array in the large chromosome. Although *rstR* typing of VCH35 revealed the presence of both El Tor and classical type *rstR*, the localization of these *rstR* alleles in the multiple CTX prophages was not confirmed. Various combinations of CTX prophage arrays have been documented, but the RS1-CTX prophage array appears to be the most frequently reported arrangement in altered El Tor strains associated with cholera outbreaks (Lee et al., 2009). Fig. 4 provides a diagrammatic comparison of various CTX prophage arrays, including the classical, prototypic seventh pandemic El Tor, and altered El Tor.

One of the main issues accompanying the emergence of atypical El Tor is standardization of the nomenclature and the classification scheme used when referring to these variants. This is further complicated by the fact that the current genotypic and phenotypic diversity reported among atypical El Tor strains is only the tip of the iceberg, because there are frequent new reports. In 2009, Lee et al. proposed the classification of atypical El Tor strains into two groups based on genetic differences in their RS1 element and the CTX prophage structure on each chromosome. Group I represents atypical El Tor strains with a tandem repeat of classical CTX prophage on the small chromosome, while Group II represents those possessing the RS1 and CTX prophage with El Tor type *rstR* and classical *ctxB* on the large chromosome. Based on these criteria, the Matlab and Mozambique variants were classified into Group I, while altered El Tor, such as those described by Nguyen et al. (2009), fell into Group II. This classification system was also used by Goel et al. (2011) to categorize the VCH35 isolate from Hyderabad, India into Group I, because it carried a tandem repeat of CTX prophage on the small chromosome. The type of *rstR* gene determines the type of corresponding CTX prophage, so a minor discrepancy when adhering to this classification system arises when both El Tor and classical type *rstR* are present, as is the case with VCH35. Therefore, the exact nature of the CTX prophages in tandem arrangements needs to be elucidated to ascertain whether VCH35 truly belonged to Group I. The existence of VCH35 also questions the possible need for a subgroup within Group I, should further analysis of VCH35 reveal the presence of both an El Tor and classical type CTX prophage in a tandem arrangement. In-depth genetic analysis of VCH35 is highly warranted before any conclusions can be drawn on its classification. In contrast, only one array of the RS1-CTX prophage has been reported in Group II (Lee et al., 2009). Therefore, we were able to describe a new type of array belonging to Group II based on the findings of this study. An arrangement of RS1 in tandem repeats followed by CTX prophage with an El Tor type *rstR* and classical *ctxB* was characterized in this study. This suggests that more varieties of the CTX prophage array may exist among the altered El Tor than are currently known.

The current study revealed an El Tor type *rstR* in the CTX prophage, but we also reported in our previous study that the 2009 Kelantan outbreak strain carried the El Tor type *tcpA* gene allele. In order to substantiate the El Tor lineage of this strain, we performed additional PCR analysis on the repeat in the toxin (RTX) gene cluster. The RTX gene cluster in *V. cholerae* was first identified and characterized in 1999 and it was found to be physically linked to the downstream region of the CTX prophage. The RTX gene cluster consists of *rtxA*, *rtxB*, *rtxC*, and *rtxD* genes, and it is responsible for the cytotoxic activity of *V. cholerae* in mammalian cells *in vitro*. However, gene deletions in the RTX gene cluster (specifically the *rtxC* gene and the downstream region of the *rtxA* gene) were noted among the classical biotype of *V. cholerae* O1, which resulted in defective production of cytotoxic activity (Lin et al., 1999). Based on this observation, Chow et al. (2001) developed a PCR assay targeting the *rtxC* gene

for biotyping *V. cholerae* serogroup O1. We used this biotyping assay and found that all the isolates possessed the *rtxC* gene. This provided further evidence that the El Tor genomic backbone was preserved in the Kelantan altered El Tor strain.

The emergence of multidrug-resistant *V. cholerae* strains has been documented frequently in recent years (Kiiru et al., 2009; Jain et al., 2011) and this phenomenon has led to repeated calls for the more prudent use of antibiotics by the global community. Multidrug-resistant *V. cholerae* have been reported from Malaysia and the antibiogram profile of the Kelantan outbreak strain showed that the isolates were resistant to various antibiotics, including, tetracycline, erythromycin, sulfamethoxazole-trimethoprim, streptomycin, penicillin G, and polymyxin B. However, they were susceptible to ciprofloxacin, norfloxacin, chloramphenicol, gentamicin, and kanamycin (Ang et al., 2010). Therefore, we investigated the phenotypes of the outbreak strain by characterizing the corresponding genes encoding for antibiotic resistance. Amita et al. (2003) studied antibiotic resistance genes in *V. cholerae* O1 and showed that the class I integron carrying the *aadA1* gene cassette was prevalent in strains isolated before 1992, whereas the SXT element was prevalent in strains isolated after 1992. Integrons are characterized by the presence of an integrase gene (*intI*) that mediates recombination between the *attI* site found on the integron and the *attC* site on the gene cassette. The insertion of a gene cassette into the integron results in the expression of functional proteins using a promoter found in the integron (Recchia & Hall, 1995). In agreement with Amita et al. (2003), PCR analysis of the Kelantan outbreak strain showed it was negative for the class I integron and the gene cassette *aadA1* encoding resistance to streptomycin and spectinomycin.

In one of the most remarkable events in the recorded history of cholera, a novel serogroup of *V. cholerae* emerged in 1992 that was designated O139 and it replaced the O1 El Tor biotype in Bangladesh and the Indian subcontinent where it became the dominant strain, although its reign was short-lived (Faruque et al., 2003). The *V. cholerae* serogroup O139 differed from serogroup O1 in having a different somatic antigen and it was also uniquely characterized by its antibiotic resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin. Interestingly, a distinctive pattern of antibiotic resistance was found after the re-emergence of serogroup O1 El Tor in 1994 where all the El Tor strains were found to be resistant to these four antibiotics, which strikingly resembled the profile of serogroup O139 (Waldor et al., 1996). The corresponding antibiotic resistance genes in serogroup O1 El Tor were collectively referred to as ICE $V_{ch}$ Ind1 (previously known as SXT<sup>ET</sup>) and they were genetically closely related to the SXT<sup>MO10</sup>, which encodes for antibiotic resistance to the same four antibiotics mentioned above by serogroup O139. Based on a comparative DNA analysis, both the ICE $V_{ch}$ Ind1 and the SXT<sup>MO10</sup> elements were considered to be derived from a common precursor (Hochhut et al., 2001; Burrus et al., 2006). The results of PCR conducted on the SXT constin in this study showed that all the Kelantan outbreak strains contained the SXT element. Genes conferring resistance to sulfamethoxazole (*SullI*), trimethoprim (*dfrA1*), and streptomycin (*strB*) were detected in all isolates, with the exception of the chloramphenicol resistance gene (*floR*). The detection of the antibiotic genes was consistent with the findings in the phenotypic antibiotic susceptibility testing and the SXT constin detected in the present outbreak strain appeared to have a deletion of the *floR* gene, when compared with the STX<sup>ET</sup> reported from elsewhere (Hochhut et al., 2001). The SXT constin without the *floR* gene represented a variant of the SXT<sup>ET</sup> constin and, other than this study,

the SXT variant has also been found among altered El Tor strains from India (Goel & Jiang, 2010). It was recently reported that an altered El Tor strain carrying both an integron and an SXT element had been identified among outbreak strains from Solapur, India (Jain et al., 2011). These findings are important, because the management of cholera patients usually entails fluid replacement therapy to replace the electrolytes lost during profuse diarrheal bouts. However, antibiotic therapy serves as an adjunct to fluid replacement therapy to reduce the duration of the disease and the excretion of the bacterium (Lindenbaum et al., 1967). Thus, continuous monitoring of changes in antibiotic resistance patterns is highly recommended, because the SXT constin harbouring various antibiotic resistance genes can be acquired easily via lateral gene transfer (Iwanaga et al., 2004).

## 5. Conclusion

Genetic analysis performed on the Kelantan altered El Tor strain isolated during a cholera outbreak in 2009 revealed a novel CTX prophage array where a tandem repeat of the RS1 element was found upstream of the CTX prophage on the large chromosome. This is the first report of a RS1-RS1-CTX prophage array among altered El Tor strains that fit into Group II according to the classification system of Lee et al. (2009). All isolates carried the SXT constin and we identified genes conferring resistance to sulfamethoxazole, trimethoprim and chloramphenicol, which correlated with their phenotypic expression in the antibiogram profile.

The emergence of the altered El Tor is viewed as an evolutionary optimization of *V. cholerae* strains in the development of a successor to the current cholera pandemic. The altered El Tor strains are poised to become epidemiologically dominant and they might hold the key to sustaining the current seventh pandemic. Equipped with the unique characteristics of the classical and El Tor biotypes, the altered El Tor has already been associated with more severe cases of cholera (Siddique et al., 2010) and it appears to be widely disseminated around the globe. The research community should actively unravel the wealth of knowledge that lies within these atypical El Tor strains and gain a better understanding that can be translated into measures to combat and conquer cholera.

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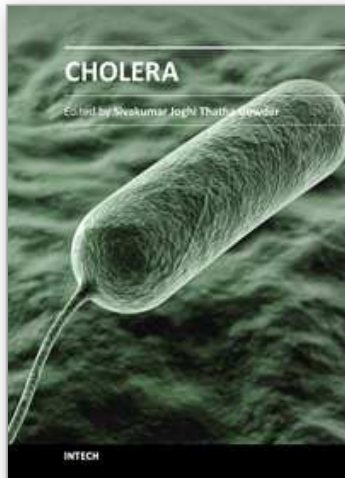
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## **Cholera**

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Cholera, a problem in Third World countries, is a complicated diarrheal disease caused by the bacterium *Vibrio cholerae*. The latest outbreak in Haiti and surrounding areas in 2010 illustrated that cholera remains a serious threat to public health and safety. With advancements in research, cholera can be prevented and effectively treated. Irrespective of "Military" or "Monetary" power, with one's "Own Power", we can defeat this disease. The book "Cholera" is a valuable resource of power (knowledge) not only for cholera researchers but for anyone interested in promoting the health of people. Experts from different parts of the world have contributed to this important work thereby generating this power. Key features include the history of cholera, geographical distribution of the disease, mode of transmission, *Vibrio cholerae* activities, characterization of cholera toxin, cholera antagonists and preventive measures.

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