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Original Article

MLST/MVLST Analysis and Antibiotic Resistance of *Vibrio cholerae* in Shandong Province of China

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Abstract

Background: *Vibrio cholerae* is an important bacterium causing profuse watery diarrhea. Cholera had swept the whole Shandong province from 1975 to 2013.

Methods: From epidemiological data and pulsed-field gel electrophoresis data, we selected 86 *V. cholerae* isolates appearing in Shandong Province in China from 1975 to 2013 and characterized them by multilocus sequence typing (MLST)/multi-virulence locus sequence typing (MVLST), antibiogram and analysis of genes related to antibiotic resistance.

Results: Combined MLST/MVLST data revealed 33 sequence types and a major group. Within the group, 3 subgroups (ST1, ST24 and ST29) were revealed, prevalent in the strains isolated during the 1980s, 1990s and 21st century, respectively. All the O1 isolates after 1990 were found to be El Tor variants harboring the classical *ctxB* gene. The *tcpA* gene of O139 strains had a mutation at amino acid position 62 (N→D). Antibiotic resistance of *V. cholerae* increased over time. Most El Tor variants between 1998 and 1999 were resistant to trimethoprim/sulfamethoxazole. The O139 strain, since its appearance in 1997, had significantly broader spectrum of antibiotic resistance than O1 variants. The presence of the SXT element corresponds to the trend of growing drug resistance.

Conclusion: The analysis of genotypic polymorphism and enhanced resistance of *V. cholerae* indicated continuous variation and evolution of this pathogenic agent in Shandong Province.

Keywords: *Vibrio cholerae*; Shandong; Antibiotic resistance

Introduction

Vibrio cholerae is the causative agent of cholera, a life-threatening diarrhea disease that remains a serious public health issue, particularly in developing countries. To date, more than 200 serogroups of *V. cholerae* have been recognized on the basis of the somatic O antigen. However, only the O1 and O139 serogroups are responsible for epidemic and pandemic cholera in humans. The O1 serogroup is classified into 2 biotypes, classical and El Tor. Both biotypes are further divided into 2 major serotypes, Ogawa and Inaba. Seven cholera pandemics have been recorded since its emergence in 1817. The ongoing seventh pandemic is considered to have begun in

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1961 in Indonesia and was caused by the O1 El Tor biotype (1). At the end of 1992, serogroup O139 was recognized in Bangladesh and India and subsequently spread to many Asian countries (2). Recently, new variants of *V. cholerae* O1 El Tor with traits of the classical biotype have appeared and become predominant globally (3, 4).

In 1964, cholera caused by *V. cholerae* O1 El Tor was introduced into Shandong, a coastal province of Eastern China. From our routine surveillance, almost every district of Shandong Province has experienced a cholera epidemic from 1975 to 2013 and 2 multiyear cholera epidemics resulting in significant morbidity were distinguished (5). One was mainly caused by serotype Inaba during 1980-1989, and the other was caused by serotype Ogawa during 1993-1999. Of note, serotype Inaba re-emerged and initiated a small cholera outbreak in 2001. O1 El Tor strains have been undetected since 2002. Conversely, serogroup O139 was first isolated in Shandong Province in 1997 and has been sporadic since then.

Pulsed field gel electrophoresis (PFGE) is a highly discriminating subtyping method for epidemiology investigations, but it requires strict compliance with the standard protocol (6). Multilocus sequence typing (MLST) and multi-virulence locus sequence typing (MVLST) utilize nucleotide sequence data for large-scale screening of bacterial populations and are easy to perform and compare by different laboratories (7).

In this study, we used a combined MLST/MVLST method to determine the overall genetic relatedness of *V. cholerae* isolates from the Shandong province of China from 1975 to 2013. The antibiotic resistance patterns against 25 different antibiotics as well as the mechanisms of antimicrobial resistance were also investigated to extend the characterization of these isolates.

Materials and Methods

V. cholerae representative strains and DNA extraction

From epidemiological data and pulsed-field gel electrophoresis data, 86 representative isolates were selected from Shandong Province during 1975-2013. We included 8 environmental strains as controls. Among these 86 isolates, 47 strains belonged to serotype Ogawa, 27 strains belonged to serotype Inaba, and 12 strains belonged to serogroup O139. Bacterial chromosomal DNA was extracted from a single colony grown overnight on nutrient agar by using the DNeasy Blood & Tissue kit (QIAGEN, Germany).

Combined MLST/MVLST and sequence analysis

Combined MLST/MVLST analysis may improve the discriminatory power and is suitable for the local epidemiological study of *V. cholerae* (7). Six housekeeping genes (*gyrB, purH, pyrC, metE, pncB* and *fumC*) and 3 virulence genes (*ctxAB, tcpA* and *toxR*) were selected for the combined MLST/MVLST analysis in Table 1 (7, 8). PCR conditions for the housekeeping genes consisted of 95 ºC for 5 min (pre-denaturation) followed by 35 cycles of 94 ℃ for 30 sec (denaturation), 55 ℃ for 30 sec (annealing) and 72 ℃ for 1 min (extension) and a final extension at 72 ℃ for 5 minutes. PCR amplification was performed to detect the 3 virulence genes with the same protocol as above except for annealing temperature at 59 ℃. The PCR products were sequenced by using the Automated DNA Sequence Analyzer ABI3730 (Applied Biosystems). Sequences were aligned by using the BioEdit program. Sequences with at least one nucleotide difference were assigned arbitrary allele numbers, with '0' for no amplification and '1' for the allele at each loci of N16961 strain. Numbers for sequence types (STs) were assigned for the 6 housekeeping genes and the 3 virulence genes of all tested strains. On the basis of the allelic difference among isolates of above-mentioned 9 genes, a minimum spanning tree was constructed by using BioNumerics (Applied Maths).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for 25 antibiotics, including amoxicillin/clavulanic acid, cephalothin, cefoxitin, cefuroxime, ceftriaxone,

ceftazidime, cefixime, cefepime, ampicillin, nalidixic, amikacin, norfloxacin, ciprofloxacin, levofloxacin, chloramphenicol, furazolidone, gentamicin, kanamycin, tetracycline, doxycycline, trimethoprim/sulfamethoxazole, azithromycin, erythromycin, imipenem and polymyxin, was carried out using broth microdilution method. *Escherichia coli* ATCC 25922 was the reference quality control strain in antibiotic susceptibility tests and results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (9).

Table 1: Primers used in this study

Gene	Primer sequences (5'~3')		Size (bp)
ctxAB	F: GGCTGTGGGTAGAAGTGAAACGG	R: CTAAGGATGTGGAATAAAAACATC	1080
tcpA	F: CACGATAAGAAAACCGGTCAAGAG	R: CGAAAGCACCTTCTTTCACGTTG	453
toxR	F: CCTTCGATCCCCTAAGCAATAC	R: AGGGTTAGCAACGATGCGTAAG	713
gyrB	F: TGGTTTTTGAGGTGGTGGAT	R: CGCTTGATTCTTACGGTTAC	1214
purH	F: TCAGCGTATCAGATAAAACC	R: CTTGCCATTCACCACACTCC	1083
pyrC	F: TGACAACACTGACGATTACA	R: TTGCGAGGTAGGCCGTAGAA	910
metE	F: TTGCTCTTGAAAAATACTGG	R: GAGAAGACTTACGCGCAACA	1110
pncB	F: GTTTTCTCCCCACATCATTC	R: TTCACACATGGCTTTTTCCG	1174
fumC	F: TTATCAAGCACAAACTCAAC	R: TCACAGGCAGCATCACATTA	1013
int I	F: GTTCGGTCAAGGTTCTG	R: GCCAACTTTCAGCACATG	923
int IV	F: GTGTTCGCGAATTTATGC	R: ACGGGATAA TGGGCTTAA	936
sxt	F: ATGGCGTTATCAGTTAGCTGGC	R: GCGAAGATCATGCATAGA CC	1035

F: forward primer, R: reverse primer.

PCR screening of antibiotic resistance genes

All strains were screened for the presence of the class I and IV integrons and SXT element by using the primers listed in Table 1. PCR cycling conditions were 95 ℃ for 5 min, then 35 cycles (94 ℃ for 30 sec, 55 ℃ for 1 min, 72 ℃ for 1 min) followed by a final extension at 72 ℃ for 7 minutes.

Statistical methods

Data analysis involved use of SPSS 17.0 (Chicago, IL, USA). The difference in antibiotic resistance between two serogroups of strains was assessed by one-way ANOVA. $P<0.05$ was considered statistically significant.

Results

Nucleotide diversity of each gene and sequence types generated by MLST/MVLST

We sequenced 9 nucleotide fragments of the genes for each of the 86 *V. cholerae* isolates. The numbers of alleles were 2 for *tcpA*, 3 for *ctxAB*, while 6-9 for *toxR* and the 6 housekeeping genes (Fig. 1). This range in number of alleles suggests that the primary virulence genes of *V. cholerae*, *tcpA* and *ctxAB* possessed relatively minor diversity as compared with the 6 housekeeping loci.

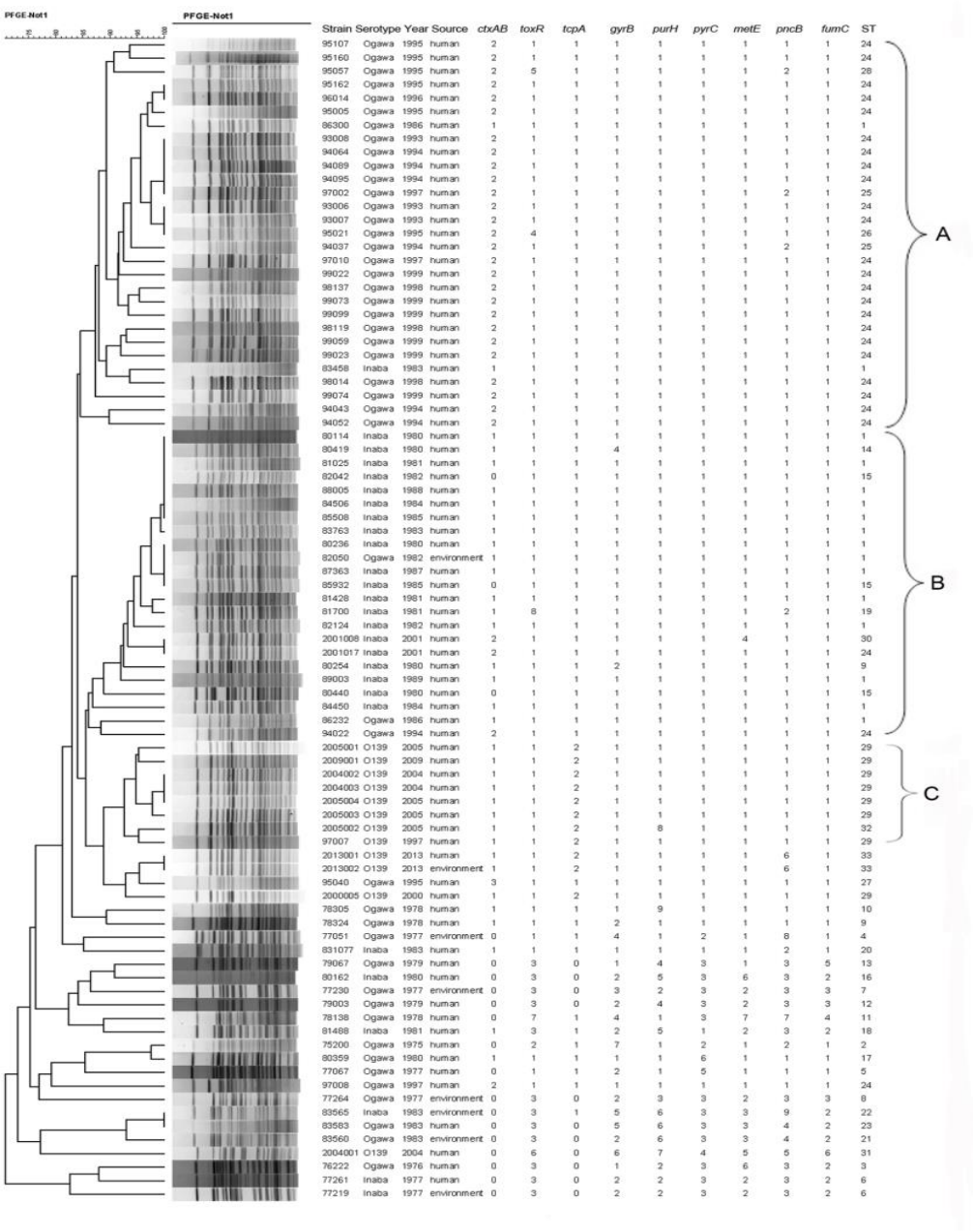

Fig. 1: Pulsed field gel electrophoresis dendrogram (*Not I)* and allele profiles at each locus obtained from 86 *V. cholerae* strains occurring in Shandong province from 1975 to 2013.

The representative strain of the seventh pandemic, N16961, was used as a control and designated ST1. Overall, 33 different sequence types were identified among the 86 isolates from Shandong province (Fig. 1). The most common allelic profile was ST24, containing 25 Ogawa strains and one Inaba strain, followed by ST1, which contained 13 Inaba strains and 3 Ogawa strains. The third major sequence type was ST29, which included 8 O139 strains.

Genetic relatedness of MLST/MVLST sequence types among V. cholerae strains

For the minimum spanning tree of the 9 genes (Fig. 2), 81.4% (70/86) of the *V. cholerae* strains clustered in one major group highlighted in light orange owing to one or 2 allelic differences. Overall, the group contained 95.9% (70/73) of the strains appearing during 1980-2013, which indicates their close relationship since 1980. On the basis of nucleotide differences, the major group contained 3 subgroups of closely related STs (ST1, ST24 and ST29), all differing by one gene. ST1 (16 isolates) and ST24 (26 isolates) predominated in the strains isolated during the 1980s and 1990s, respectively, corresponding to the 2 multiyear epidemics. Most O139 isolates (ST29) were closely related to ST1 as compared with ST24. Of the O139 isolates, 2004001 clearly differed from most O139 clinical isolates in each gene and appeared to be unrelated to the main group of 70 strains. Of note, O139 strains isolated in 2013 had a distinct allelic profile to the dominant O139 subgroup, with one housekeeping gene difference.

Antimicrobial resistance profile of V. cholerae strains

The antimicrobial susceptibility of the 86 *V. cholerae* strains was determined against 25 antimicrobial agents (Table 2). Among the *V. cholerae* isolates, resistance to polymyxin was the most common (82/86, 95.3%), followed by erythromycin (70/86, 81.4%), and imipenem (33/86, 38.4%). All *V. cholerae* strains before 1980 were susceptible to the remaining 22 antimicrobial agents except for 7.1% to 14.3% of strains that were intermediately resistant to cefoxitin, amoxicillin/clavulanic acid, gentamicin and norfloxacin. In 1983, one environmental strain resistant to ampicillin and another clinical strain resistant to norfloxacin were identified. The prevalence of resistant strains increased during the multiyear epidemic in the 1990s. In the 21st century, although only sporadic cholera cases and localized outbreaks occurred, resistance to the tested antibiotics was higher. The strains from this period were 100% resistant to polymyxin and erythromycin. At the same time, high rates of resistance (>50%) to ampicillin, nalidixic, gentamicin, tetracycline, kanamycin, imipenem and trimethoprim/sulfamethoxazole were detected.

Characterization of genetic elements associated with antibiotic resistance

Spread of antibiotic resistance in *V. cholerae* has been attributed to the mobilization of integrons and a novel conjugative transposable element, SXT (10). The distribution of class I and IV integrons and SXT element in *V. cholerae* of Shandong province was examined by PCR. All tested stains carried the *int IV* gene, which indicates the presence of the superintegron regardless of serotype and years of isolation. The SXT element was present in Ogawa strains during 1998-1999 and all the O139 strains since 1997.

PCR detection of *int I* revealed that 29% (25/86) of *V. cholerae* strains were positive for the class I integron. All the 21 O1 Ogawa strains obtained from 1993 to 1997 carried the class I integron. In addition, only 25% (3/12) of O139 strains contained the class I integron. Another *int I*-positive strain belonged to the Inaba serotype and was isolated in 1983. The class I integron of *V. cholerae* strains in Shandong province was first identified in the early 1980s.

Table 2: Antibiotic resistance profiles of 86 *V. cholerae* strains

Antibiotic	***Resistance percentages (%)***				
	1975-1979	1980-1989	1990-1999 (32.3%)[a]	2000-2013 (76.9%)[a]	Total
Cephalothin	.0	.0	3.2	8.3	2.3
Cefoxitin	.0	.0	3.2	.0	1.2
Cefuroxime	.0	.0	9.7	8.3	4.7
Ceftriaxone	.0	.0	3.2	8.3	2.3
Ceftazidime	.0	.0	3.2	.0	1.2
Cefixime	.0	.0	3.2	.0	1.2
Cefepime	.0	.0	3.2	.0	1.2
Ampicillin	.0	3.4	.0	66.7	10.5
Amoxicillin/clavulanic Acid	.0	.0	3.2	16.7	3.5
Trime-thoprim/sulfamethoxazole	.0	.0	25.8	50.0	16.3
Chloramphenicol	.0	.0	.0	41.7	5.8
Furazolidone	.0	.0	6.5	16.7	4.7
Nalidixic	.0	.0	3.2	75.0	11.6
Ciprofloxacin	.0	.0	.0	8.3	1.2
Levofloxacin	.0	.0	.0	8.3	1.2
Amikacin	.0	.0	3.2	.0	1.2
Gentamicin	.0	.0	3.2	66.7	10.5
Kanamycin	.0	.0	9.7	75.0	14.0
Tetracycline	.0	.0	6.5	58.3	10.5
Doxycycline	.0	.0	6.5	33.3	7.0
Azithromycin	.0	.0	6.5	16.7	4.7
Erythromycin	64.3	89.7	74.2	100.0	81.4
Imipenem	35.7	37.9	29.0	66.7	38.4
Polymyxin	85.7	96.6	96.8	100.0	95.3
Norfloxacin	.0	3.4	3.2	.0	2.3

[a] SXT positive percentages

Discussion

In our previous study, the PFGE database of 250 *V. cholerae* isolates from 1975 to 2013 has been established (5). The predominant serotype of Shandong province from 1975 to 2013 changed from Ogawa (1975-1979) to Inaba (1980-1989), Ogawa (1993-1999), Inaba (2001), then O139 (1997-2013). We found that spatiotemporal serotype shifts generally correlated with the variations in the PFGE patterns (5). In each stage, representative strains showing both a dominant PFGE pattern and other diverse pulsotypes were included to achieve the greatest genetic diversity. At least one strain was selected from each outbreak. Overall, 86 representative isolates were selected from Shandong province during 1975-2013. Figure 1 shows PFGE profiles (*Not I*) obtained from 86 *V. cholerae* strains isolated in Shandong province from 1975 to 2013.

Different housekeeping genes were used for molecular typing of *V. cholera* (7). Several reported methods were used by our research and had poor discrimination for pathogenic cholera strains in

the seventh pandemic (7, 11). According to Salim's study, we selected 6 genes with sequence variation in the toxigenic isolates (12). These 6 genes had higher diversity in choleragenic strains from Shandong Province from 1975 to 2013. Four of the 6 housekeeping genes (*gyrB, purH, metE* and *fumC*) are spaced around the large chromosome (chromosome I) of *V. cholerae*, and the remaining (*pncB* and *pyrC*) are located on chromosome II. Our data revealed extensive housekeeping genetic diversity among cholera isolates during 1975-1980. Since 1980, 80.8% of cholera strains showed the same allelic pattern as N16961 in the 6 housekeeping genes. The low variability of the 6 housekeeping genes since 1980 indicated that all the 6 loci evolved at a slow rate in the past 40 years.

Cholera toxin (CT) and the toxin coregulated pilus (TCP) are the principal virulence factors encoded by *ctxAB* and *tcpA* (13). The *toxR* gene codes for a transcriptional activator regulating CT gene expression and TCP biogenesis (14). These 3 virulence genes were included in the study to increase the discrimination of MLST analysis. *CtxAB* is composed of 2 subunits, *ctxA and ctxB*, and heterogeneity within the *ctxB* gene serves as the basis for *ctxB* genotyping (7). Our data illustrated that *ctxB* gene in O1 El Tor isolates during the 1990s and 2001 (ST24-28 and ST30) carried 2 non-synonymous substitutions relative to N16961. These 2 changes are characteristic of *ctxB* in classical strains of the sixth pandemic, resulting in amino acid substitution at positions 39 and 68 of the *ctxB* gene. Therefore, the strains possessing the allelic type 2 and 3 in *ctxAB* are El Tor variants, replaced the prototype seventh-pandemic O1 El Tor biotype in Shandong Province since 1993. The complete substitution of El Tor variants for the prototype El Tor in Shandong province occurred about 10 years earlier than that in other provinces (15) reflecting the local characteristics of *V. cholerae* in Shandong province. The evolution and emergence of new pathogenic variants of *V. cholerae* O1 in Shandong province agrees with several reports from Asia, Africa, and America (16, 17).

The minimum spanning tree resulting from analysis of sequences of all loci showed that almost all the isolates from 1980 to 2013 formed a homogenous group. Within the group, 3 sets of subgroups were revealed, designated ST1, ST24 and ST29. As shown in Fig. 2, ST1 subgroup with identical ST to O1 El Tor N16961 comprised mainly the isolates of serotype Inaba and predominated in the first multiyear epidemic in the 1980s, indicating that the progenitor isolate was likely to be imported from Bangladesh. ST24 subgroup, in which most isolates were El Tor Ogawa variants, was prevalent in the second multiyear epidemic during the 1990s. Cholera in Shandong Province was entirely caused by O139 strains since 2002. Most O139 isolates belonged to ST29, closely related to 3 other O139 strains (ST32 and ST33). O139 strains in these 3 genotypes were most similar to O1 El Tor strains with the allelic profile of ST1, in keeping with previous finding that the O139 serogroup evolved from the El Tor biotype, with major variations in the O-antigen (7). ST29 differed from ST1 at the *tcpA* locus. The *tcpA* gene of O1 strains in Shandong Province showed 100% homology with that of N16961. However, the *tcpA* gene of O139 strains from Shandong Province had a mutation at amino acid position 62 (N→D). A similar change of *tcpA* gene was also found in O139 strains isolated from other provinces of China (GenBank accession no. AF512409-AF512413). From the variation in one housekeeping gene (*pncB*), one O139 strain from seafood and one O139 strain from a patient in 2013 were distinguished from other O139 isolates previously identified, sharing a new allelic pattern, ST33. From PFGE analysis, these strains in 2013 showing a unique pulsotype were also separated from the O139 isolates occurring in the past 10 years and predominated in China in recent years, suggesting the risk for human infections with ST33 from seafood in Shandong province. MLST/MVLST genotypes ST1, ST24 and ST29 matched PFGE groups B, A and C, and serotypes Inaba, Ogawa and O139, respectively. Both methods revealed a larger variability of *V. cholerae* strains in the early stage. The MLST method

showed that all toxigenic O139 isolates from 15 provinces in China had the same ST as the El Tor N16961 strain (13). However, MLST/MVLST described in this study distinguished O139 strains (ST29-33) from N16961 strain (ST1). Additionally, numerous unique sequence types were observed with the MLST/MVLST approach, which demonstrates the high discriminatory capability of this approach. The chosen 9 genes appear to be well suitable for broader population structure studies of *V. cholerae*.

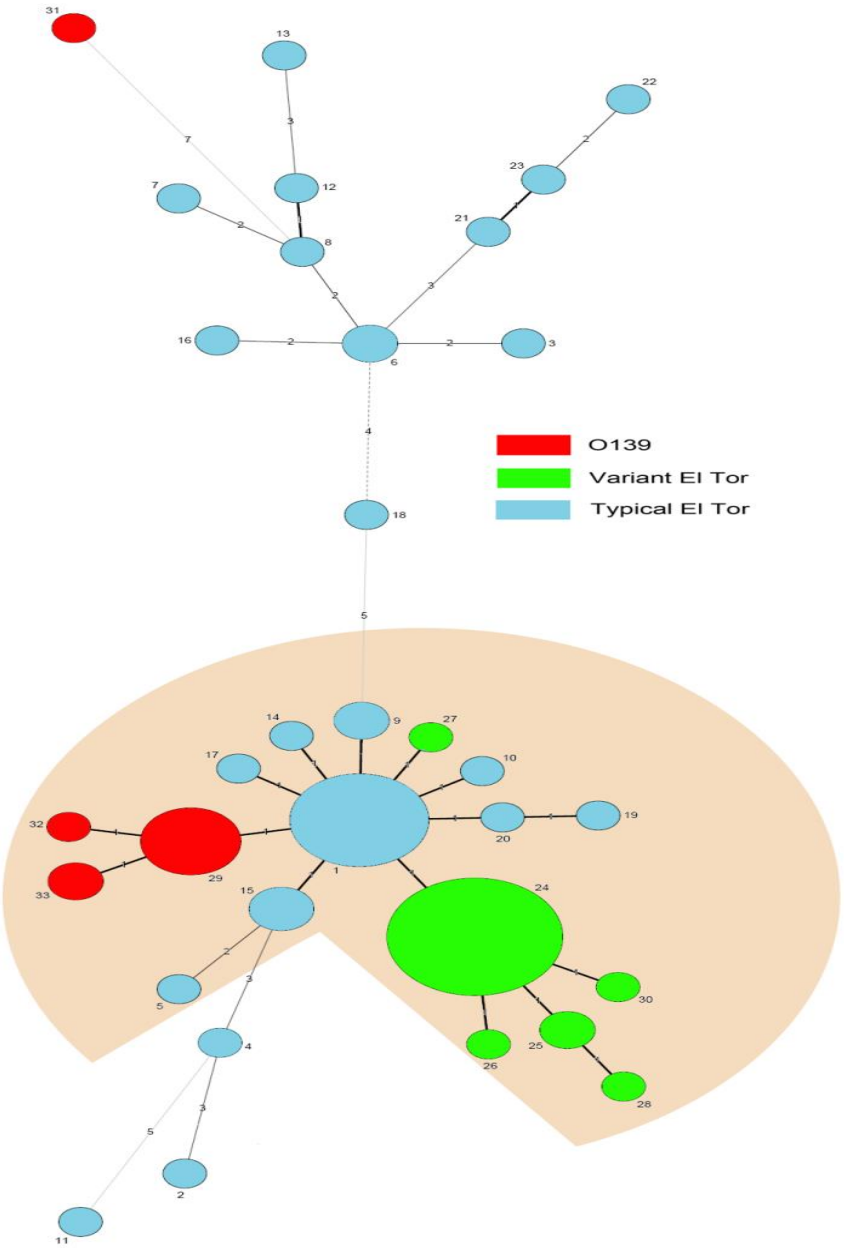

Fig. 2: The minimum spanning tree based on sequence types from combined multilocus sequence typing and multivirulence locus sequence typing. The number at the node represents the sequence type (ST), and the size of the node reflects the number of isolates belonging to the same ST. The width of the line between each node corresponds to the number of allelic changes between STs in the middle part of this line. The part in light orange indicates the major group with one or 2 allele differences

Antibiotics are often used to reduce the duration and symptoms of diarrhea. In this study, we surveyed antimicrobial resistance profiles among *V. cholerae* strains isolated in Shandong province from 1975 to 2013 using a panel of 25 antibiotics. Increased antibiotic resistance was observed over time. The resistance profiles suggest that polymyxin, erythromycin and imipenem were not effective for treating O1 and O139 *V. cholerae* infections from 1975 to 2013. For O1 Ogawa variants emerging in the 1990s, most antibiotics remained useful, except for trimethoprim/sulfamethoxazole, which had a high rate of resistance, 25.8% (Table 2). Accordingly, SXT elements were detected in O1 Ogawa variants between 1998 and 1999, and 77.8% of which were resistant to trimethoprim/sulfamethoxazole. Meanwhile, SXT elements were not detected in O1 variant strains isolated in 1993-1997 and 2001 as well as all O1 prototype strains susceptible to trimethoprim/sulfamethoxazole, which indicates the association of the presence of the SXT element and resistance to trimethoprim/sulfamethoxazole (18). In short, antimcrobial resistance of the O1 El Tor isolates from the current seventh pandemic in Shandong province is not as severe as that described in other counties (19).

For O139 strains, resistance to 13 antibiotics, including ampicillin, gentamicin, tetracycline, kanamycin, chloramphenicol, azithromycin, and trimethoprim/sulfamethoxazole, increased to the greatest extent. The resistance rate remained <10% for only ciprofloxacin, levofloxacin, amikacin and cephalosporin antibiotics. The emergence of resistance to azithromycin and doxycycline in a few O139 strains is of great concern because these new generations of antibiotics are usually sufficient to reduce the symptoms of cholera and stabilize the disease (20). The class I integron was mainly distributed among strains of *V. cholerae* O1 between 1993 and 1997, and only one O1 strain in 1983 carried the class I integron, which agrees with the regional characteristics of O1 El Tor epidemic clones in China (21). As well, 25% of the toxigenic *V. cholerae* O139 strains in Shandong province were positive for the class I integron. Therefore, the class I integron had a limited role in the drug resistance of both O1 and O139 strains from Shandong province, which disagrees with previous investigations in China (22). Of interest, O139 strains had significantly broader spectrum of antibiotic resistance than O1 variant Ogawa strains during 1998-1999, although both types of *V. cholerae* carried the SXT element (F=13.4, P<0.01). With horizontal gene transfer, SXT elements have been found in almost all clinical cholera strains since their identification in 1996 (23). Antibiotic genes located at variable regions of SXT elements are unstable and diverse (24).

Further study of antibiotic genes in SXT elements may help in understanding the mechanisms of the extensive resistance of O139 strains.

Conclusion

The 6 housekeeping loci and 3 virulence loci chosen in this study constitute a suitable basis for MLST/MVLST typing, enhancing the understanding of molecular epidemiology and evolution of *V. cholerae* in the ongoing seventh pandemic in Shandong province. We demonstrated the occurrence of *V. cholerae* O1 variants in Shandong Province for the first time. The increase in rate of resistance in O1 variants and especially O139 strains highlights the need for continuous surveillance of antibiotic susceptibility and provides important information for the control of *V. cholerae* infections.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interests.

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