

Original Article

Study of drug resistance and molecular typing of 59 cholerae01 clinical isolates from 1984 to 2002 in Chongqing, China

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Abstract: Objective: To analyze the correlation between drug resistance and Cholerae01 clinical isolates from 1984 to 2002 in Chongqing, China. Methods: K-B assay was applied to detect the sensitivity of 59 Cholerae01 clinical isolates (20 Ogawa, 39 Inaba) to 16 kinds of antibiotics. BioNumerics software was used for a cluster analysis of electrophoresis patterns obtained from the Not I enzyme-cutting genomic DNA by Pulsed-field gel electrophoresis (PFGE). Results: *Vibrio cholerae*01 in Chongqing area, China were highly resistant to Cotrimoxazole, Furazolidone and Streptomycin. The resistance rates were 28.81% (17/59), 61.02% (36/59) and 30.51% (18/59), respectively. While the isolates from the crowd were sensitive to Amikacin, Gentamicin, Tobramycin, Ampicillin, Neomycin and Doxycycline, and no drug-resistant strains were observed. Conclusion: No significant changes are found in the drug resistance of *Vibrio cholerae*01 from the crowd in Chongqing, China and the drug resistances of the Ogawa and the Inaba strains are different. *Vibrio cholerae*01 from the crowd in Chongqing, China are highly homologous, which may be from the epidemic strains with the same source.

Keywords: *Vibrio cholerae*01, drug-resistance, molecular typing

Introduction

Cholera is defined as category A infectious disease in China on the Prevention and Treatment of Infectious Diseases and it is one of international quarantine infectious diseases. There were 7 pandemics of *Vibrio cholerae*01 in the world, causing huge losses to human's lives and properties. All of Cholera epidemic strains in Chongqing, China before 2002 were *Vibrio cholerae*01, therefore, we studied the Pulsed-field gel electrophoresis (PFGE) molecular typing and drug resistance of *Vibrio cholerae*01 clinical isolates in Chongqing from 1984 to 2002, analyzed the genetic correlation and drug resistance change of *Vibrio cholerae*01 in Chongqing, China at the molecular level, as well as the epidemic regularity. It is crucial to develop effective prevention and control measures and provide basic knowledge for prevention and clinical rational drug use to the disease in the region.

Previous studies have suggested that PFGE molecular typing of *Vibrio cholerae* and drug resistance may be result of the single genetic event occurrence for the strains with the same source, such as gene mutation, insertion, deletion [1]. The relationship between *Vibrio cholerae*01 strains in Chongqing and *Vibrio cholerae*01 strains in other regions around the country need further studies. Some of other countries and regions have established their own PulseNet [1, 2]. In this study, clinical samples of *Vibrio cholerae*01 in Chongqing, China were isolated from 1984 to 2002. The sensitive of drug resistance for these samples were analysis. 21 Ogawa and 40 Inaba *Vibrio cholerae*01 strains were divided into 18 subtypes, 13 PFGE types. The similarity value of the 59 *Vibrio cholerae*01 clinical isolates from the patients was about 88-100%. Our results showed that *Vibrio cholerae*01 in Chongqing, China were highly resistant to Cotrimoxazole, Furazolidone and Streptomycin. However, these

Drug resistance of 59 cholerae01 clinical isolates

Table 1. Serotype, isolated date, drug resistance spectrum and PFGE typing of the 59 *Vibrio cholerae*01 strains

Serotype	Strains No.	Isolated Date	Drug resistance spectrum	PFGE Typing
Inaba	203	2002	-	G1
Inaba	168	2002	FR	G1
Inaba	169	2002	FR	G1
Ogawa	176	2002	SXT-NOR-S	J
Inaba	191	2002	FR	G1
Inaba	192	2002	FR	G1
Inaba	128	2002	FR	G1
Inaba	129	2002	-	G1
Inaba	130	2002	FR	G1
Inaba	137	2002	FR-PIP	G1
Inaba	149	2002	FR	G1
Inaba	150	2002	FR	G1
Inaba	151	2002	FR	G1
Inaba	152	2002	FR	G1
Inaba	153	2002	FR	G1
Inaba	154	2002	FR	G1
Inaba	155	2002	-	G1
Inaba	166	2002	FR	H
Inaba	167	2002	FR	H
Inaba	38	2002	-	G1
Inaba	40	2002	-	G1
Inaba	41	2002	-	G1
Inaba	52	2002	FR	H
Inaba	53	2002	FR	H
Inaba	54	2002	-	H
Inaba	55	2002	-	H
Inaba	56	2002	FR	H
Inaba	57	2002	FR	H
Inaba	80	2002	FR	H
Inaba	82	2002	FR	H
Inaba	84	2002	-	H
Inaba	86	2002	-	H
Inaba	87	2002	-	H
Inaba	5	2001	-	G2
Inaba	15	2001	FR	G2
Inaba	20	2001	FR	G2
Inaba	188	2001	-	G2
Ogawa	175	1999	S	A1
Ogawa	135	1999	FR-S	A1
Ogawa	146	1999	SXT-FR-S	D2
Ogawa	43	1999	SXT-S	A1
Ogawa	44	1999	SXT-FR-S	A2
Ogawa	45	1999	SXT-FR-S	A2
Ogawa	46	1999	SXT-S	D1
Ogawa	47	1999	SXT-S	D2

samples were sensitive to Amikacin, Gentamicin, Tobramycin, Ampicillin, Neomycin and Doxycycline. These investigations suggest that *vibrio cholerae*01 isolated from the patients in Chongqing, China are highly homologous, which may be from the epidemic strains with the same source.

Materials and methods

Source of strains

*Cholerae*01 strains were isolated from the Cholera outbreaks in Chongqing, China from 1984 to 2002. The molecular weight standard for PFGE analysis was *Salmonella* H9812 standard strain. The quality control strain ATCA-25922 for antibiotic drug sensitivity test were provided by National Institutes for Food and Drug Control.

Reagents and instruments

Alkaline peptone water, agar 4 are provided by Beijing Land bridge Technology Co., Ltd; Drug sensitive slips and MH agar (OXOID company, UK); Cholera "O" polyvalent diagnostic mixed serum, Ogawa, Inaba diagnostic serum were purchased from Chengdu Institute of Biological Products and Ningbo Tianrun Biological Products Co., Ltd.. The photometer for the determination of the concentration of culturable bacteria (A value) was produced by BioMerieux Vitek Colorimeter Co., Ltd.

Experimental design

All the strains were isolated, identified and typed in accordance with Cholera Prevention and Treatment Handbook, and there were saline controls for serum agglutination test.

Drug sensitivity test: K-B agar disk diffusion method recommended

Drug resistance of 59 cholerae01 clinical isolates

Ogawa	32	1999	SXT-FR-S	A1
Ogawa	33	1999	SXT-FR-S	E2
Ogawa	34	1999	SXT-FR-S	B
Ogawa	42	1999	SXT-S	F2
Ogawa	13	1999	SXT-FR-S	I
Ogawa	14	1999	SXT-S	A1
Ogawa	205	1998	SXT-FR-S	C
Ogawa	143	1998	FR	C
Ogawa	6	1998	SXT-FR-S	F1
Ogawa	24	1998	SXT-FR-S	C
Ogawa	35	1998	SXT-FR-S	C
Ogawa	36	1998	-	C
Ogawa	172	1992	-	E1
Inaba	171	1984	-	K

Note: 16 antibiotics. AK: Amikacin, CN: Gentamicin, TOB: Tobramycin, AMP: Ampicillin, TE: Tetracycline, E: Erythromycin, PB: Polymyxin B, C: Chloramphenicol, SXT: Sulphamethoxazole/trimethoprim 1:19, FR: Furazolidone, NOR: Norfloxacin, PIP: pipemidic acid, CIP: Ciprofloxacin, N: Neomycin, BO: Doxycycline hydrochloride, S: Streptomycin. "-" indicates that the strain is sensitive to the 16 of testing antibiotics, and there is no drug resistance.

by WHO was applied, and the results were determined in accordance with Implementation Standards of Antimicrobial Sensitivity Test, 2006 Edition (M100-S16), and each batch of tests used the quality control ATCA25922 strain for quality control.

Pulsed-field gel electrophoresis (PFGE) Typing PFGE standard method recommended by U.S. Centers for Disease Control and Prevention was applied for the PFGE Typing of 62 Vibrio cholerae01 strains [3, 4].

Image analysis and Results-clustering Using BioNumerics Version 4.0 software, Dice correlation coefficient and UPGMA method were selected for the processing and cluster analysis of PFGE.

PFGE typing methods: Those with the same patterns after enzyme digestion were designated as the same type, such as A-type, those with 1~3 different bands compared with A-type were designated as subtypes (such as A1, A2, ... An); those with more than 3 different bands compared with A-type were designated other types, such as B-type, C-type, D-type. BioNumerics V4.0 was applied for the cluster analysis of the data by UPGMA method, and clustering similarity coefficient (distance) was calculated by the bands-comparison based Dice [5, 6].

Results

Cholerae01 strains

Based on the irregular, sporadic, year, source and other epidemic characteristics and strains typing features, a total of 59 Cholerae01 representative strains was selected for isolation and identification, analysis of drug resistance and PFGE typing, in which, 20 strains were Ogawa, and 31 strains were Inaba. Serotype, isolated date, drug resistance spectrum and PFGE typing of the 59 Vibrio cholerae01 strains were showed in **Table 1**.

Antibiotic sensitivity tests

In the drug resistance tests for 16 kinds of antibiotics, 72.88% (43/59) of the 59 Cholerae01 representative strains were resistant to more than one antibiotic, and 20.34% (12/59) were resistant to more than three kinds of antibiotics. The 59 Cholerae01 isolates from the crowd were highly resistant to Cotrimoxazole, furazolidone and streptomycin, and the resistance rates were up to 28.81% (17/59), 61.02% (36/59) and 30.51% (18/59), but sensitive or moderately sensitive to the remaining 13 kinds of antibiotics. For the 39 strains of Inaba vibrio cholerae to Furazolidone, the resistance rate was 58.97% (23/39), and for the 20 strains of Ogawa vibrio cholerae to Cotrimoxazole, Furazolidone and Streptomycin, the resistance rates were 85.00% (17/20), 75.00% (15/20) and 95.00% (19/20, **Table 1**).

PFGE typing

After Not I restriction enzyme digestion and gel electrophoresis of the genomic DNA for 59 strains of Vibrio cholerae01, the DNA fragments obtained a good separation, and the number of electrophoretic bands for each strain was 20~30. The fragments with the largest molecular weight were at about 700 kb position, and there were 10 fragments with the same molecular weights at 20~80 kb position for most of the strains, while the main differences lied at 100~700 kb position (**Figure 1**). BioNumerics software was used for a cluster

Drug resistance of 59 cholerae01 clinical isolates

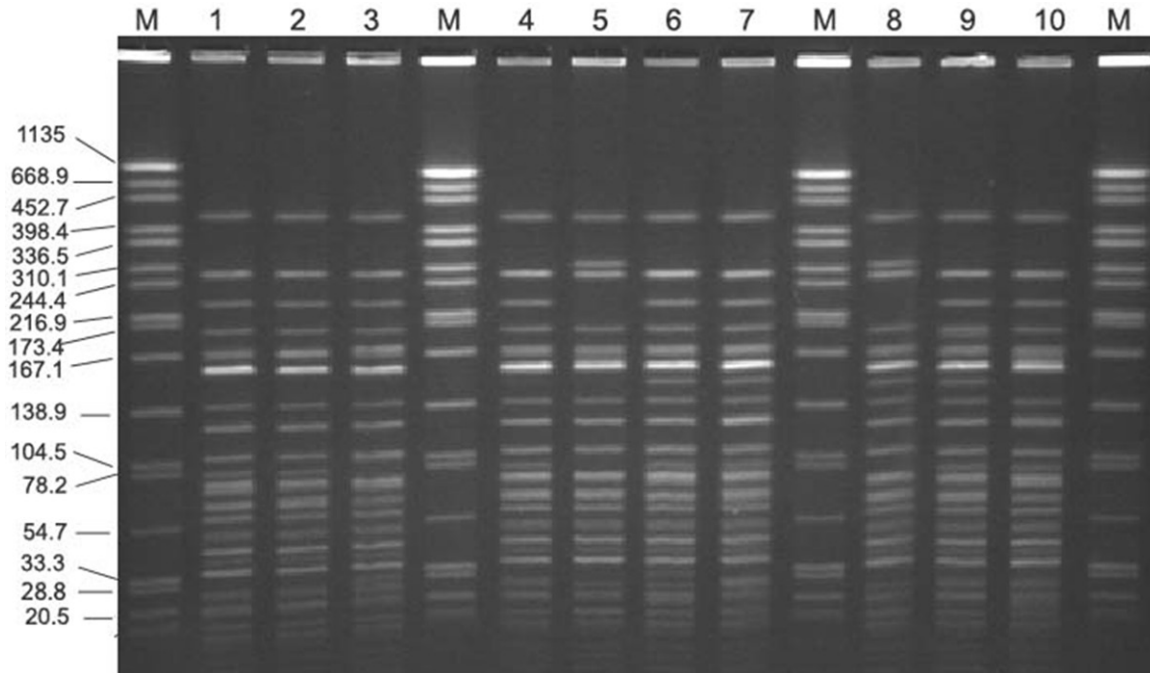


Figure 1. Pulsed-field gel electrophoresis of *Vibrio cholerae*01 after Not I enzyme digestion. Genomic DNA of 10 strains of *Vibrio cholerae*01 (Lane 1 to Lane 10) were isolated by Pulsed-field gel electrophoresis and Not I enzyme digestion. The number of electrophoretic bands for each strain was 20~30 pb and the main differences laid at 100~700 kb position. (M: marker of molecular weight).

analysis of electrophoresis patterns obtained from the Not I enzyme-cutting genomic DNA by Pulsed-field gel electrophoresis (PFGE), 59 *Vibrio cholerae*01 strains were divided into 11 kinds of PFGE genotypes A~K, those with the same number of DNA fragments and molecular weights were designated as the same subtype, and 11 genotypes could be further divided into 16 subtypes (**Figure 2**, **Table 1**). 20 Ogawa *Vibrio cholerae* isolates were divided into 8 PFGE typing, 11 subtypes (A1, A2, B, C, D, E1, E2, F1, F2, I, J), and 39 Inaba *Vibrio cholerae* isolates were divided into 3 PFGE typing, 4 subtypes (G1, G2, H, K). In 1999, 14 Ogawa *Vibrio cholerae* isolates were divided into 6 PFGE typing, 8 subtypes (A1, A2, B, D1, D2, E2, F2, I). In 1998, 6 Ogawa *Vibrio cholerae* isolates were divided into 2 PFGE typing (C and F1) (**Figure 2**). The cluster analysis of electrophoresis patterns for the 59 strains of *Salmonella typhi* by Bionumerics software found that their similarity value was about 88%~100% (**Figure 2**).

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Discussion

With the extensive application and abuse of antibiotics, the drug resistance phenomenon of *Vibrio cholerae* is worsening. A long-term drug resistance surveillance and analysis of drug resistance trends for *Vibrio cholerae* has a very important practical significance, which can timely guide clinicians for the reasonable selection of sensitive antimicrobial drugs and provide a scientific basis to prevent cholera, and find drug resistance trends of *Vibrio cholerae* and further study the mechanisms at the same time. The results of drug sensitivity monitoring for *Cholerae*01 clinical isolates in Chongqing, China from 1984 to 2002 indicate that *Vibrio cholerae*01 in Chongqing area are highly resistant to Cotrimoxazole, Furazolidone and Streptomycin, the resistance rates are 28.81% (17/59), 61.02% (36/59) and 30.51% (18/59), respectively. Nineteen strains of *Vibrio cholerae*01 with multiple antimicrobial resistances, in which, the drug resistance spectrum for

Drug resistance of 59 cholerae01 clinical isolates

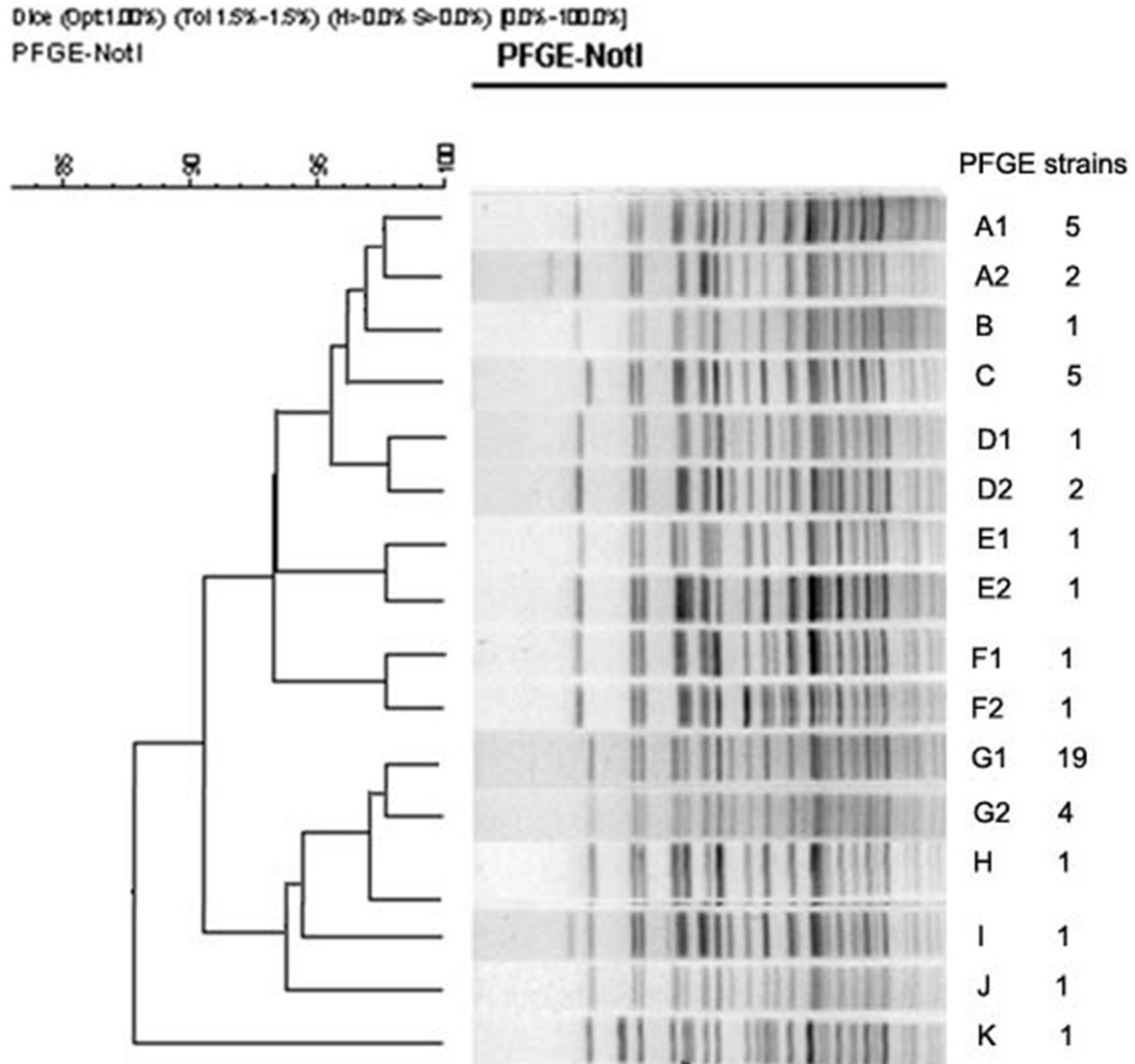


Figure 2. Pulsed-field gel electrophoresis (PFGE) typing for 59 strains of *Vibrio cholerae*01. BioNumerics software was used for a cluster analysis of electrophoresis patterns obtained from genomic DNA by PFGE. 59 *Vibrio cholerae*01 strains were divided into 11 kinds of PFGE genotypes A~K, those with the same number of DNA fragments and molecular weights were designated as the same subtype, and 11 genotypes could be further divided into 16 subtypes.

11 strains is cotrimoxazole - furazolidone - streptomycin (SXT-FR-S) resistance. *Cholerae*01 clinical isolates are sensitive to Amikacin, Gentamicin, Tobramycin, Ampicillin, Neomycin and Doxycycline, and no drug-resistant strains are observed. Although the serotypes were different and the number varied greatly for the strains monitored each year, in general, drug resistance spectrum for the different serotypes of *Vibrio cholerae*01 monitored in Chongqing are different, for example, Inaba *Vibrio cholerae* is highly resistant to Furazolidone mainly, while Ogawa *Vibrio cholerae* is highly resistant

to Cotrimoxazole, Furazolidone and Streptomycin, and both the Ogawa and Inaba are highly resistant to Furazolidone (58.97%-75.00%), the mechanism for different serotypes with different drug resistance spectrums needs further studies. From the perspective of each year, no significant changes in drug resistance of the isolates are observed; the strains isolated from some years are not statistically analyzed due to the relatively small numbers.

The drug resistance of *Vibrio cholerae*01 in Chongqing, China is not the same with the

reports in other regions, Wang Linna et al reported that the drug resistance of *Vibrio cholerae*01 in Wenling, Zhejiang to chloramphenicol, tetracycline, doxycycline, norfloxacin was worsening, and the resistance rates are up to 85%, 85.71%, 100% and 85.71% [7], while this study indicates that *Vibrio cholerae*01 in Chongqing are all sensitive or partially moderate-sensitive to chloramphenicol, tetracycline and doxycycline, and the resistance rate to norfloxacin is only 1.69% (1/59). Xia Xin et al reported that the resistance rates of *Vibrio cholerae*01 in Hunan to amikacin, ciprofloxacin and erythromycin were 100%, 100% and 72.20% [8], but those in Chongqing, China are all sensitive or moderate-sensitive to the 4 kinds of antibiotics. Overall, the drug resistance of *Vibrio cholerae*01 in Chongqing better than the above areas, and the huge differences of drug resistance for the strains between regions deserve further studies, which may help to clarify the resistance mechanisms of *Vibrio cholerae* and the reasons for different drug resistances.

PFGE method is considered as the “gold standard” of bacterial molecular typing, providing a reliable technology to determine the genetic relationship among strains, which has a better resolution and epidemiological investigative capacity than other typing methods during the practical application of *Vibrio cholerae* typing. U.S. CDC established PulseNet of core with PFGE technology in 1996, determined PFGE standard experimental program for a variety of bacteria, and realized the networking and standardization of laboratory strain analysis and laboratory surveillance of infectious diseases in different regions. PFGE typing technology is currently the most common molecular typing method used in Cholera epidemiological investigation, in recent years, some laboratories in China apply the method for Epidemiological analysis, and its powerful features and practical value has been demonstrated.

In this study, the variation of PFGE genotypes for *Vibrio cholerae*01 is small, and the overall similarity value is about 88%~100%. 20 Ogawa *Vibrio cholerae* isolates are divided into 8 PFGE typing, 11 subtypes, in which, 14 Ogawa *Vibrio cholerae* isolates from 1999 are divided into 6 PFGE typing, 8 subtypes, although it is believed that these strains may be from the same clone

strain with the combination of epidemiological data of cholera, the frequency of gene mutation, deletion, insertion and other events for the same strain during the epidemic is still relatively high. 39 Inaba *Vibrio cholerae* isolates are divided into 3 PFGE typing, 4 subtypes, and after Not I restriction enzyme digestion and gel electrophoresis of the genomic DNA, 32 Inaba *Vibrio cholerae* were divided into 3 PFGE typing, but the homology of the 3 PFGE typing is about 96%, which suggest that the cholera in 2002 was derived from the two epidemic strains of the same clone strain. In summary, this study indicates that Cholera epidemic strains in Chongqing, China during 1984 and 2002 have a high PFGE homology (88%~100%), no obvious input or appearance of heterologous strains are observed in the epidemic strains for many years. The emerging of different PFGE types may be result of the single genetic event occurrence for the strains with the same source, such as gene mutation, insertion, deletion [1]. The relationship between Cholerae01 strains in Chongqing and Cholerae01 strains in other regions around the country need further studies. Some of other countries and regions have established their own PulseNet [1, 2]. the results of this study provide PFGE fingerprints of Cholerae01 strains in Chongqing, China and establish a good foundation for the realization of data sharing, which will help to realize active surveillance of cholera disease and tracing the source of infection in China.

Disclosure of conflict of interest

None.

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Drug resistance of 59 cholerae01 clinical isolates

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