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Microbiological and epidemiological investigation of cholera epidemic in Ukraine during 1994 and 1995

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SUMMARY

The Ukraine cholera epidemic of 1994 and 1995 was caused by *Vibrio cholerae* O1, serotype Ogawa, biotype El Tor. This epidemic was centred in the area around Respublika Krim (Crimea) and Mykolajiv, and spread to include parts of southern Ukraine. Cases of cholera occurred between September and November of 1994 and between June and October of 1995. The 32 fatalities among 1370 recorded cases (case fatality ratio, 2·3%) occurred throughout the course of the epidemic. *V. cholerae* from patients with cholera produced cholera toxin and were resistant to multiple antibiotics, though no resistance plasmids were found. Conjugation experiments suggested that resistance to multiple antibiotics may be present on a self-transmissible genetic element. Environmental sources of *V. cholerae* O1 El Tor included sewage, sea and surface water, and fresh water and marine fish. All but one of the environmental *V. cholerae* isolated during the epidemic were very similar to selected isolates from patients at the same time, supporting the role of these environmental sources in the spread of disease.

INTRODUCTION

Cholera is a diarrhoeal disease characteristically producing a severe, profuse watery diarrhoea that can cause rapid dehydration, acidosis and death [1]. Up to 80% of cases are mild or asymptomatic, leading to rapid dissemination of the disease. Recently there has been a marked increase in the incidence of cholera world-wide, especially in South America [2, 3].

Vibrio cholerae serogroup O139 [4], other *V. cholerae* serogroups, and other species of *Vibrio* [5], can produce cholera or disease similar to cholera. However, *V. cholerae* O1 biotype El Tor, the organism responsible for the seventh cholera pandemic, continues to be the most significant cause of morbidity and mortality [6]. Cholera caused by this organism was introduced into the former USSR in 1965 and subsequently caused sporadic and local outbreaks through the 1970s and into the 1980s [7]. Though most of the European region was less affected by cholera in 1995 than in previous years, the 1994 cholera epidemic in Ukraine continued into 1995, with 525 reported cholera cases and 10 deaths in 1995 [6].

Epidemiological studies have demonstrated that aquatic environments can be sources of *V. cholerae* in both sporadic and epidemic disease [8], though implementation of water and sewage treatment and

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improvements in personal hygiene have eliminated cholera in most developed countries [9]. Toxigenic *V. cholerae* have been repeatedly isolated from surface waters, sewage, patients and carriers residing in high incidence areas within the former Soviet Union, including Ukraine, during the period between 1965 and 1989 [7]. Furthermore, conditions in some surface water reservoirs were found to be suitable for the survival of these bacteria; outbreaks of cholera in the former Soviet Union in the 1970s were either waterborne or associated with consumption of seafood or dairy products contaminated with *V. cholerae*. Finfish, shellfish and crustaceans have been implicated by other investigators as a source of bacteria for cholera outbreaks, and *V. cholerae* O1 has been found in shellfish and on the skin and in the intestines of finfish [9–11].

The epidemiological and environmental factors responsible for the recurrence of cholera in Ukraine in 1994 and 1995 have not yet been reported. This study was undertaken to describe this epidemic further, to determine possible routes of transmission of the disease, and to determine the microbiological characteristics and antibiotic resistance profiles of the bacterial isolates responsible.

MATERIALS AND METHODS

Collection of epidemiological data and isolates from cases and carriers in Ukraine

Epidemiological investigations in Ukraine were coordinated by Dr V. V. Alekseenko. Data concerning the epidemiology and microbiology of cholera were collected by surveillance stations consisting of epidemiological and microbiology laboratories. Surveillance stations tested environmental sources, such as water and food, for the presence of *V. cholerae*, collected data with respect to risk factors for transmission of cholera, including food and environmental exposures, and identified contacts at risk for developing cholera. Patient data and clinical information were obtained from hospitals both during and after the epidemic and from surveillance stations, which reported to the Ukraine Ministry of Health daily during the epidemic. Bacteria were isolated from clinical samples at the local hospital laboratory using standard methods. Patient isolates of *Vibrio* spp. were collected from hospitals and stored at the Laboratory of Cholera Infection, Kiev Research Institute.

Cases of cholera were defined as a diarrhoeal illness with isolation of *V. cholerae* O1 from stools. The severity of cases was graded according to levels of dehydration as measured on the basis of clinical findings and data from laboratory analysis of blood and plasma. Loss of fluid up to 3% of the total body weight corresponds to Level I dehydration (mild disease), a 4–6% loss corresponds to Level II dehydration (moderate disease), a 7–9% loss corresponds to Level III dehydration (severe disease), and greater than a 10% fluid loss corresponds to Level IV dehydration (severe disease). Only hospitalized cases with culture confirmation of *V. cholerae* infection were included in the analysis of the epidemic. Carriers were defined as individuals culture positive for *V. cholerae* that showed no sign of overt disease. To identify carriers, surveillance stations tested individuals thought to be at risk for infection with *V. cholerae*, including children in day-care facilities, individuals working in the food industry, people living in areas with a high incidence of cholera, and contacts of previously identified cases.

Detection of cholera toxin

To identify directly cholera toxin (CT) in bacterial culture supernatants, a reverse-phase passive latex agglutination assay (VET-RPLA; Unipath Ltd., Basingstoke, UK) was used according to the manufacturer's directions. All isolates negative for CT production in the VET-RPLA when grown in buffered peptone water were tested again after overnight growth in AKI medium [12, 13].

PCR for detection of the cholera toxin gene

PCR for detecting a 564-bp region of the *ctxA* gene was done using the CTX2 and CTX3 primers according to the protocol of Fields and colleagues [14], except that the reactions were carried out for 35 cycles. The presence of amplified DNA was demonstrated by electrophoresis of reaction products through 0·8% agarose, ethidium bromide staining and visualization with long-wave ultraviolet light. Isolate 93-0608, previously characterized as CT positive, was used as a positive control.

Antibiotic susceptibility/resistance

Isolates were tested for antibiotic susceptibility in standard Kirby Bauer disk diffusion and agar dilution assays [15, 16]. Interpretation of zone diameters for the disk diffusion assay was performed according to NCCLS standards developed for *E. coli* [17] since no

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such guidelines exist for *V. cholerae*. Antibiotics tested in the disk diffusion assay were: ceftriaxone, cephalothin, ciprofloxacin, doxycycline, furazolidone, gentamicin, kanamycin, norfloxacin, ofloxacin, streptomycin, sulphamethoxazole/trimethoprim, sulphisoxazole, tetracycline. Antimicrobial susceptibility was also determined in the Vitek apparatus (bioMérieux Vitek, Inc., Hazelwood, MO, USA) according to the instructions of the manufacturer using GNS and GNS-OT Vitek antimicrobial susceptibility cards. GNS cards test for the following antibiotics: amikacin, ampicillin, carbenecillin, cefamandole, cefoxitin, cephalothin, chloramphenicol, gentamicin, tetracycline, tobramycin, sulphamethoxazole/trimethoprim. GNS-OT cards test for: amoxicillin, ampicillin, carbenecillin, ceftriaxone, cephalothin, ciprofloxacin, gentamicin, nalidixic acid, nitrofurantoin, norfloxacin, ofloxacin, tetracycline and sulphamethoxazole/trimethoprim. The agar dilution method was used to confirm susceptibility or resistance to ampicillin, tetracycline, nitrofurantoin and chloramphenicol. Finally, the susceptibility or resistance of some isolates was checked by growth of bacteria on Mueller–Hinton agar containing appropriate concentrations of antibiotics.

Plasmid analysis

Plasmids were isolated by the method of Birnboim and Doly [18], Kado and Liu [19] or Eckhardt [20] and separated by horizontal electrophoresis using 0·5–1·0% (w/v) agarose gels (Gibco BRL *ultra*PURE, Life Technologies Inc., Gaithersburg, MD, USA) and TBE buffer (0·089 M Tris, 0·089 M boric acid, 0·002 M EDTA). Gels were stained with ethidium bromide and photographed under u.v. transillumination. Assessment of the megaplasmid content of vibrio isolates was also done according to the methodology of Barton and colleagues [21].

Conjugation experiments

Conjugations were carried out on Mueller–Hinton agar plates according to the method of Waldor and colleagues [22]. *V. cholerae* isolates described in this study were used as donors and *Escherichia coli* XL1-Blue MR (Stratagene Inc., La Jolla, CA, USA) as recipient. Transconjugants were selected on Mueller–Hinton agar plates containing the appropriate antibiotic (trimethoprim, 50 μg/ml; streptomycin, 10 μg/ml; cefoxitin, 16 μg/ml; carbenecillin, 16 μg/ml; ampicillin, 25 μg/ml; trimethoprim, 32 μg/ml with sulphamethoxazole, 160 μg/ml) to select against the recipient strain and nalidixic acid (50 μg/ml) to select against donor strains.

RESULTS

Epidemiology of the epidemic

Ukraine had a relatively low incidence of cholera for many years after the outbreak of 1970–1, with the exception of a small outbreak of *V. cholerae* El Tor serotype Inaba in 1974 (Fig. 1). The period of extremely low incidence of cholera in 1978–90 was followed by an outbreak in the southern part of Ukraine in 1991 (Odeska, Khersonska and Mykolajivska oblasts) [23]. Few cases of cholera were seen again until 1994–5, when Ukraine experienced a dramatic resurgence in disease caused by *V. cholerae* El Tor strains (Fig. 1). Serotype Ogawa predominated during this epidemic, comprising 2365/2369 (99·9%) of all isolates from patients and carriers in 1994 and 1995. Most cases were concentrated in or near Mykolajiv (Table 1), though cholera was also found in the Respublika Krim (Crimea) and the Khersonska, Odeska, Dnipropetrovska and Donentzka oblasts. With the exception of the latter two oblasts, most cases occurred along or near the coast of the Black Sea with only a scattered few cases found farther inland (Fig. 2). There was a very low rate of carriage of *V. cholerae* in the population in the years preceding the 1994–5 epidemic (Fig. 1), though carriage did rise slightly in 1993 (Fig. 1, Table 1). No cases of cholera have been reported in Ukraine in 1996 or 1997.

The first cases of cholera were seen in the Respublika Krim between 1 September and 5 September 1994 (see Fig. 3*a*). During this period there was a shortage of treated drinking water in this area due to drought. At the same time, surveillance stations reported contamination of the drinking water in one area of Simferopol with untreated sewage due to influx of water from sewage supply pipes into drinking water supply pipes. Cholera appeared in neighbouring Mykolajivska oblast during the period 6–10 September 1994, peaked 16–25 September 1994, and declined rapidly thereafter. In 1994 89% (757/845) of the Ukrainian cholera cases occurred in September (see Fig. 3*a*). Carriers of *V. cholerae* were found by the second week of the epidemic and continued to be identified until the beginning of November. Fatalities occurred throughout the peak of the epidemic period. In 1994, there were no recorded cases of cholera or

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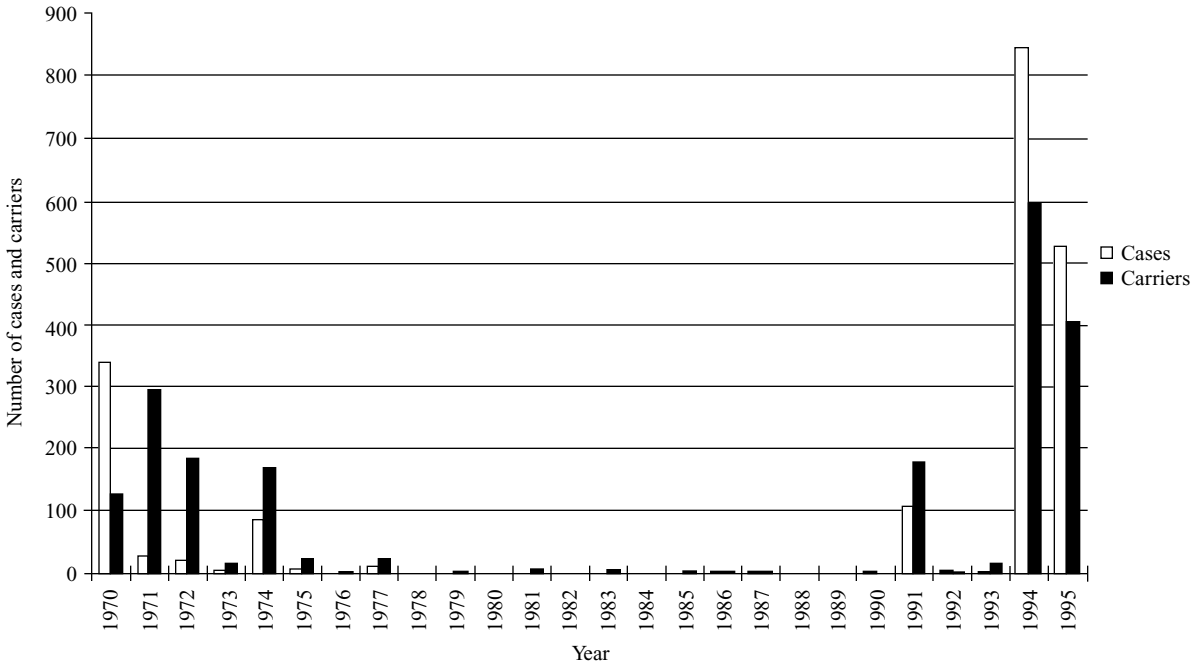

Fig. 1. Cases of cholera and carriers of *V. cholerae* in Ukraine, 1970–95. The Figure is an adaptation of the data of Narkevich and colleagues [23], with additional data collected by V. V. Alekseenko.

Table 1. *Geographical distribution of cholera cases and* V. cholerae *carriers in Ukraine, 1991–5*

	Year*				
Oblast	1991	1992	1993	1994	1995
Cherkaska	0 (0)	0 (0)	0 (0)	2 (2)	0 (0)
Chernivetzka	0 (0)	0 (0)	0 (0)	1 (0)	2 (7)
Dnipropetrovska	1 (0)	0 (0)	0 (0)	36 (11)	0 (0)
Donetzka	1 (0)	0 (0)	0 (0)	21 (45)	0 (2)
Khersonska	10 (19)	0 (0)	1 (0)	68 (49)	10 (21)
Ivano-Frankivska	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)
Kirovogradska	0 (0)	0 (0)	0 (0)	2 (7)	3 (0)
Luganska	0 (0)	0 (0)	0 (0)	2 (1)	0 (0)
Lvivska	0 (0)	0 (0)	0 (0)	3 (1)	0 (0)
Mykolajivska	53 (39)	0 (0)	0 (1)	512 (410)	467 (319)
Odeska	41 (115)	1 (1)	2 (12)	5 (9)	41 (49)
Respublika Krim (Crimea)	0 (0)	1 (0)	0 (0)	182 (29)	0 (0)
Rovenska	0 (0)	0 (0)	0 (0)	0 (1)	0 (0)
Sevastopol	0 (0)	0 (0)	0 (0)	1 (7)	0 (0)
Zaporizska	0 (6)	0 (0)	0 (0)	9 (28)	0 (0)
Zytomirska	0 (0)	0 (0)	0 (0)	1 (0)	1 (0)

* Numbers of cases are indicated for each region, with numbers of carriers in parentheses.

carriers of *V. cholerae* detected in Ukraine after 9 November.

In 1995, 525 cholera cases occurred between 2 June and 4 October, appearing first in Mykolajivska oblast and later in Khersonska and Odeska oblasts, with a few sporadic cases in other areas. Disease incidence was spread throughout the warmer months of 1995 (Fig. 3*b*), with 193 cases in June, 164 in July, 65 in August, 99 in September, and the remaining 4 cases between 30 September and 4 October of 1995. No

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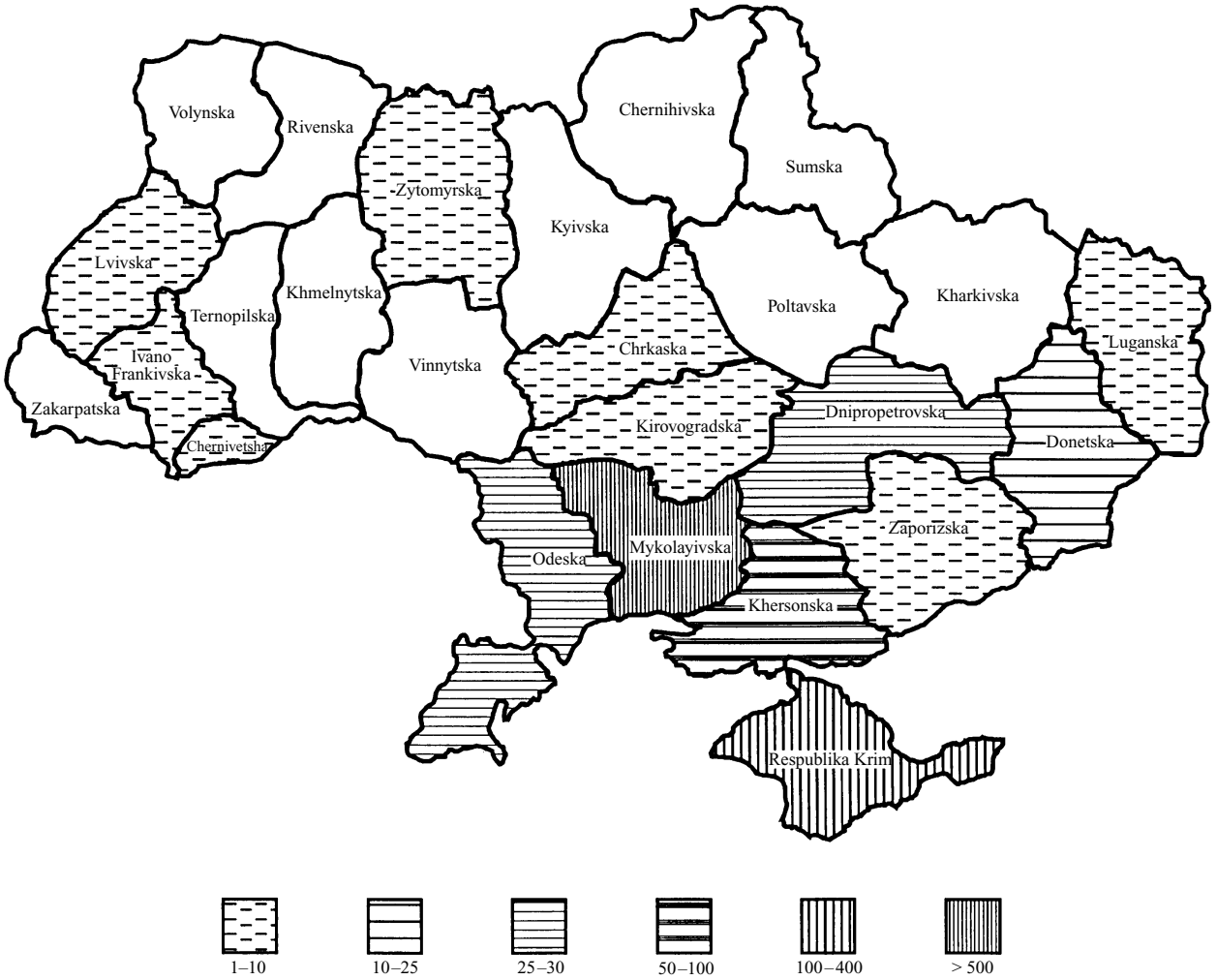

Fig. 2. Geographical distribution of cases of cholera in Ukraine during the epidemic of 1994–5. The numbers of cases in each oblast are indicated by the fills in the boxes below the map.

cholera has been reported in Ukraine after 4 October 1995.

As can be seen from Table 1 and Figure 2, the main focus of infection during the epidemic was in the Mykolajivska oblast. Data for 1995 indicate that cholera was more prevalent in the city of Mykolajiv (64 % of cases) than in towns or rural areas of this oblast (36 % of cases). Surveillance stations analyzed between 2 and 200 samples per day from environmental sources, food and humans, depending on the location of the station and the epidemic situation. Infection in rural regions was usually associated with foodborne transmission occurring during large gatherings of people, such as weddings or parties. In several cases, some of the individuals who prepared food for these gatherings were from Mykolajiv and were found to be carriers of *V. cholerae*. Carriers were also found among other groups: children in day-care facilities, individuals working in the food industry, people living in areas with a high incidence of cholera, and contacts of previously identified cases. Overall, 600 carriers were identified in Ukraine in 1994 and 399 were identified in 1995.

Of the cases of cholera in Mykolajiv and neighbouring regions for which transmission factors could be identified 51 % were associated with consumption of the marinated fish dish made with *Cluponella*, a member of the fish family *Clupeidae* (herring). The onset of cholera symptoms occurred between 1 and 72 h after consumption of this fish dish in the 57 cases for which these data exist, with symptoms appearing at 24 h in 47/57 (82 %) of cases. Examination of 135 cases in Kherson of acute diarrhoea caused by bacteria other *V. cholerae* (*Salmonella* spp., *Shigella* spp., etc.) showed no association with ingestion of *Cluponella*. *Cluponella* was fished from sewage-containing waters

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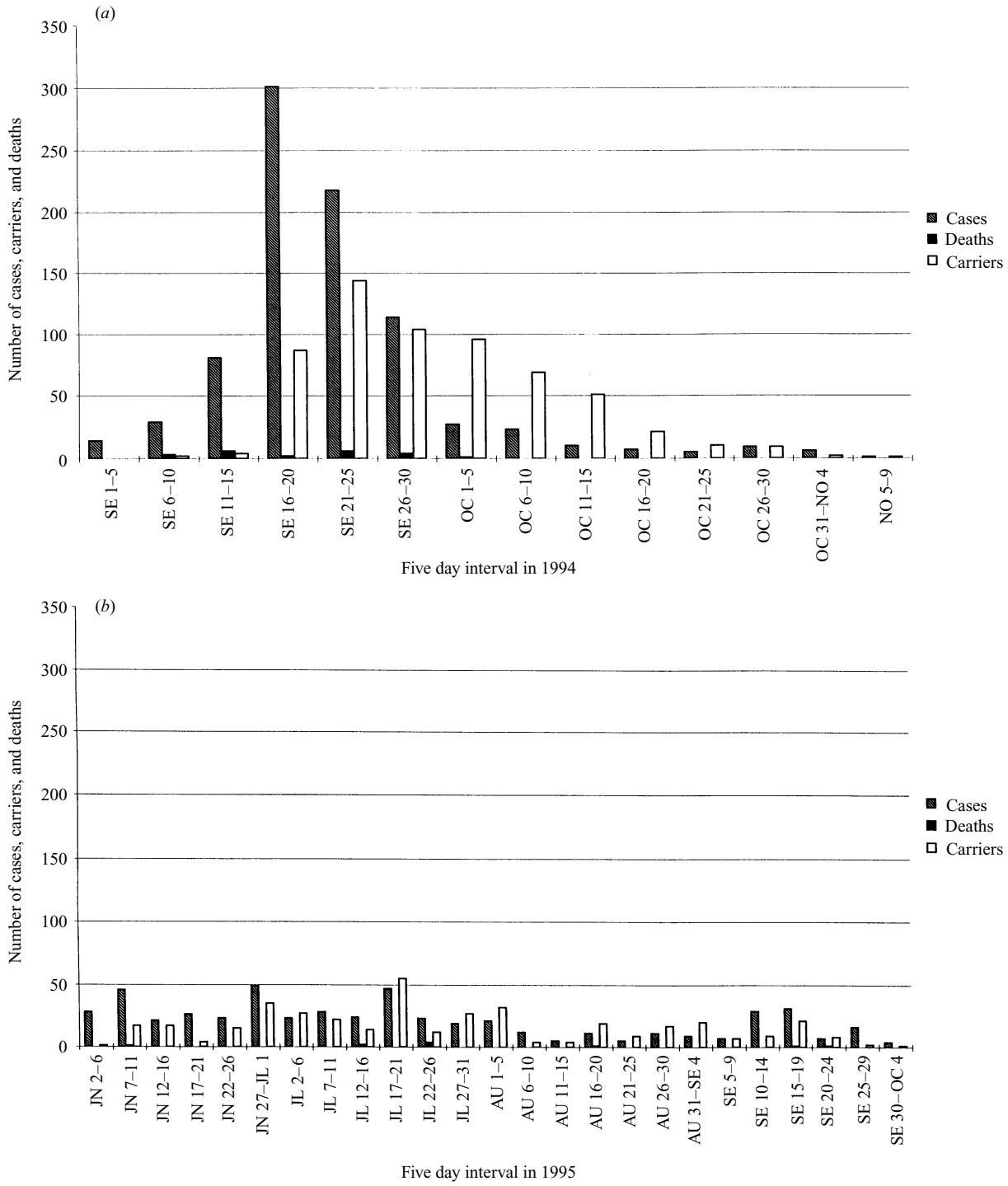

Fig. 3. Cholera cases, deaths due to cholera, and carriers of *V. cholerae* for each 5-day period during the 1994–5 Ukrainian cholera epidemic. (*a*) Data for 1994, beginning on 1 September and ending 9 November. (*b*) Data for 1995, beginning 2 June and ending 4 October.

contaminated with *V. cholerae* in areas near Mykolajiv, sold in Mykolajiv, and distributed to other areas in the region. In two cases, bacteriologists succeeded in isolating *V. cholerae* O1 from fish suspected of causing cholera infection. These isolates, however, were not available for further characterization.

Of the 1370 recorded cases of cholera during the 1994–5 epidemic, 720 patients were male and 650 were female, giving a male:female ratio of 1·2:1. Ad-

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ditionally, 186 (13·6%) of the 1370 patients were less than 20 years of age, 321 (23·4%) were between 20 and 40 years of age, 525 (38·3%) were between 40 and 60 years of age, and 338 (24·7%) were 60 years of age or more.

Clinical findings and antibiotic treatment

Most cases were typical of cholera, with large volumes of stool, dehydration and further symptoms requiring hospitalization, though the degree of dehydration varied. Severe cholera (as defined in the Materials and Methods) accounted for 25·5% of the cases in 1994 and 25% of the cases in 1995, while moderate cholera accounted for 34·3% of the cases in 1994 and 35·7% of the cases in 1995. Mild cholera was diagnosed in 40·2% of the cases in 1994 and 39·3% of the cases in 1995. Rehydration therapy was undertaken in hospitals upon diagnosis of cholera.

There were 22 fatalities in 1994, 10 in Mykolajivska oblast and 12 in Respublika Krim. Of the 10 fatalities in 1995, 8 occurred in Mykolajivska oblast and 2 occurred in Odeska oblast. Overall there were 32 fatalities among 1370 recorded cases, a 2·3% case fatality rate. If carriers are also included, there were 32 fatalities among 2369 cases and carriers (1·4% fatality rate). The ages of the 32 patients who died from cholera ranged from 2 months to 90 years, with a mean of 52 ± 21 years. Nineteen of the patients who died were male and 13 were female, giving a male : female ratio of 1·5 : 1.

Patients were treated with a number of antibiotics during the epidemic, including chloramphenicol, tetracycline, erythromycin, doxycycline, gentamicin and trimethoprim/sulphamethoxazole. Early in the epidemic carriers were treated with doxycycline and chloramphenicol, but these were discontinued when carriers were found to remain colonized after 6 days of treatment. Erythromycin and norfloxacin then became the preferred choices for treatment.

Characteristics of isolates

Forty isolates from patients with severe cholera and from environmental sources were selected for further characterization. No isolates were available from carriers. *V. cholerae* strains isolated from Ukraine between 1987 and 1995 were characteristic seventh pandemic strains having serogroup O1, serotype Ogawa, biotype El Tor when tested according to the protocols in the Materials and Methods (Table 2). Two environmental strains isolated from water in 1976 and 1987 did not produce detectable O antigen, while an environmental strain isolated in 1982 was a non-O1 *V. cholerae*.

Results from PCR analysis showed that the gene coding for CT (*ctx*) was present in all human *V. cholerae* isolates as well as all environmental isolates obtained after 1987 except one 1995 river water isolate (95-0752). A *V. cholerae* isolated from a fresh-water fish in 1993 (95-0745), just prior to the 1994–5 epidemic, also contained the *ctx* gene. With the exception of isolate 96-604, the *ctx* gene was not present in environmental isolates prior to 1988.

The VET–RPLA, was used to detect CT in culture supernatants. Two isolates, 95-0762 and 95-0777, failed to produce detectable CT though both had CT-like activity against both CHO and Vero cells that could be neutralized with specific anti-CT antiserum (data not shown). Furthermore, PCR demonstrated the presence of the *ctx* gene in both isolates. Only one environmental *V. cholerae* isolated before 1991 (96-0604) contained the *ctx* gene and produced CT detectable by the VET–RPLA test, whereas all environmental *V. cholerae* isolated after 1991, with the exception of 95-0752, contained the *ctx* gene and produced CT.

Antibiotic resistance

Most Ukrainian *V. cholerae* isolates tested in this study were resistant to three or more of the antibiotics tested (Table 2). These strains were isolated over a period of several months from a relatively large geographical area (including Mykolajiv, Odessa and Kirovograd), and were found both in humans and in the environment. After allowing for the uncertainty regarding intermediate resistances, the human and environmental isolates from the Ukrainian epidemic can be grouped into three broad categories: (1) isolates that were all sensitive to sulphamethoxazole/trimethoprim but were resistant to sulphisoxazole alone or in combination with streptomycin, furazolidone and amoxicillin, or with an intermediate resistance to cefoxitin, (2) isolates resistant to sulphisoxazole, furazolidone, streptomycin and sulphamethoxazole/trimethoprim with occasional resistances to other antibiotics, and (3) isolates resistant to sulphisoxazole, streptomycin, sulphamethoxazole/trimethoprim, amoxicillin and furazolidone, with occasional resistances to other antibiotics. The first group included environmental strains isolated before

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Table 2. *Antibiotic resistance patterns of* V. cholerae *isolates, Ukraine, 1994–5*

Resistance pattern*	Year	Source	Isolate No.
Group I			
G	1976	Surface water	96-0600
	1982	Surface water	96-0602
G, Cef^{I}	1987	Surface water	96-0601, 96-0603
G, Amx^{R}	1995	Surface water	95-0752
G, S, Cef^{I}, Fz^{R}	1994	Patient	94-0457 (Peru strain)
G, S, Amx^{R}, Cef^{I}	1987	Surface water	96-0604
Group II			
G, S, Sxt, $Fz^{I/R}$	1994	Patient	95-0755, 95-0759, 95-0761
	1995	Patient	95-0750
	1994	Surface water	95-0776
G, S, Sxt, F^{R}, $Cef^{I/R}$	1993	Patient	93-608 (Pakistan strain)
	1994	Patient	95-0762
	1993	Fish	95-0745
G, S, Sxt, E, Fz^{R}, Cef^{I}	1995	Patient	95-0747
Group III			
G, S, Sxt, $Amx^{I/R}$	1991	Patient	95-0743
	1994	Patient	95-0754, 95-0758, 95-0767, 95-0768
G, S, Sxt, $Amx^{I/R}$, $Fz^{I/R}$	1994	Patient	95-0756, 95-0760, 95-0763, 95-0769, 95-0770, 95-0781
	1995	Patient	95-0751, 95-0753
	1994	Sewage	95-0772
	1994	Sea water	95-0779, 95-0780
G, S, Sxt, $Amx^{I/R}$, Cef^{I}	1995	Patient	95-0749
G, S, Sxt, $Amx^{I/R}$, Cef^{I}, $Fz^{I/R}$	1994	Patient	95-0757, 95-0764
	1995	Patient	95-0746
	1994	Sewage	95-0771
	1994	Surface water	95-0777
G, S, Sxt, Amx^{R}, Cb^{I}, Fz^{I}	1994	Surface water	95-0775
G, S, Sxt, Amx^{R}, Cb^{I}, F^{I}, Cef^{I}	1994	Sea water	95-0773

* The abbreviations used for antibiotics are as follows: Amx, Amoxicillin/CA; Ap, Ampicillin; Cb, Carbenecillin; Cef, Cefoxitin; Cm, Chloramphenicol; E, Erythromycin; F, Furazolidone; G, Sulphisoxazole; Na, Nalidixic acid; S, Streptomycin; Sxt, Sulphamethoxazole/trimethoprim; Tet, Tetracycline; Sensitive, superscript S; Intermediate resistance, superscript I; Resistant, superscript R.

1991, the 1995 river water isolate (95-0752), and a disease-associated isolate from Peru (94-0457) included as a control strain. The second group contained 1994 isolates from Mykolajiv that were associated with mortality, 1995 patient isolates from Mykolajiv that were not associated with mortality, a fresh water fish isolate from Odeska oblast in 1993 (95-0745), and an environmental isolate from 1994. An isolate originating from Pakistan, included as a control strain, was also included in this group of isolates, suggesting that the Ukrainian isolates may share some relationships with seventh pandemic strains from other parts of the world. The third category of antibiotic resistance isolates contained organisms from both 1994 and 1995, from all geographical areas of Ukraine that were affected, and from both patients and environmental sources. All three groups of antibiotic resistance patterns were present at the same time, and there appears to have been relatively little change in antibiotic resistant phenotypes during the course of the 1994–5 epidemic. The antibiotic resistance profile of isolate 95-0752, which was isolated from river water near Odessa in 1995, was most similar to environmental isolates obtained from water

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in Ukraine from 1976–87. Antibiotic resistance profiles of these earlier environmental isolates were most similar to that of a recent isolate from Peru (94-0457). All strains were tested and found to be sensitive in the disk diffusion assay to the following additional antibiotics: ceftriaxone, cephalothin, ciprofloxacin, doxycycline, gentamicin, kanamycin, norfloxacin and ofloxacin. Strains tested using GNS and GNS-OT Vitek cards were all sensitive to amikacin, ampicillin, cefamandole, ceftriaxone, cephalothin, ciprofloxacin, gentamicin, norfloxcin, ofloxacin and tobramycin.

There were some differences in antibiograms obtained using different methods. While all isolates from Ukraine exhibited intermediate or full resistance to tetracycline when the disk diffusion assay was performed, none was found to be resistant using either the Vitek protocol with GNS and GNS–OT susceptibility test cards or the agar dilution method. Similarly, isolate 95-0747 was ampicillin resistant in the disk diffusion assay and sensitive in Vitek and agar dilution assays, and isolates 95-0746, 95-0762 and 95-0777 had intermediate or full resistance to chloramphenicol in the Vitek assays but were all sensitive in the agar dilution test. Resistance to nitrofurantoin was also variable for a number of isolates.

With the exception of a 30 kb plasmid in isolate 95-0766, the *V. cholerae* strains investigated did not contain plasmids when examined by four different methods. However, trimethoprim and streptomycin resistance was transferred by conjugation from all resistant Ukrainian isolates into the *E. coli* XL1-Blue MR cloning strain as a recipient. Resistance to each antibiotic was selected independently and, although the frequency of transfer varied slightly depending on the strain, it averaged about 5×10^{-7} transconjugants. All trimethoprim-resistant transconjugants were also resistant to streptomycin and sulphamethoxazole. Similarly, all streptomycin-resistant transconjugants were also resistant to trimethoprim and sulphamethoxazole. None of the five antibiotic-resistant transconjugants obtained from each mating contained plasmid DNA. Twenty transconjugants were tested in experiments using isolate 95-0766, which contained a 30 kb plasmid, as donor. None of the transconjugants possessed plasmid DNA. No streptomycin-resistant colonies were obtained in two independent matings when the environmental strain, 96-0604, and the Peruvian strain, 94-0457, were used in conjugation experiments.

Resistance to sulphamethoxazole, trimethoprim and streptomycin could not be conjugated from either the resistant *V. cholerae* donors 95-0743, 95-0749 and 95-0777. No cefoxitin-resistant transconjugants were obtained when any of the resistant strains was used as a donor in conjugation experiments with *E. coli* XL1-Blue MR as a recipient. Similar results were obtained with the carbenecillin-resistant isolates 95-0775 and 95-0773.

DISCUSSION

Cholera has been known in Ukraine at least since 1830, when it was introduced from Russia into Ekaterinoslav (now Dnipetropetrovsk) and spread rapidly to the more heavily populated north and central parts of the country. During 1830 and 1831, 199910 cases and 86331 fatalities were attributed to cholera. Cholera reappeared in 1847–8 (660827 cases; 232562 fatalities), in 1852–5 (197300 cases, 76732 fatalities), in 1865–73 (370003 cases; 138795 fatalities, in 1892–5 (128420 cases; 51695 fatalities), and in 1908–11 (67246 cases; 31152 fatalities) [24, 25]. Small outbreaks and sporadic cases of cholera were reported every year from the beginning of the first world war until 1923 [26]. Of the first six known world-wide cholera pandemics, therefore, only the first did not affect Ukraine. In the case of all five cholera pandemics affecting Ukraine, disease appears to have spread from neighbouring Russian regions into eastern Ukraine and from there throughout the country.

The epidemic that occurred in 1994 and 1995 in Ukraine appears to be an extension of the seventh cholera pandemic. The causative organism was *V. cholerae* O1, biotype El Tor, serotype Ogawa. Although the carriage rate for this organism rose slightly in the Odeska oblast during 1993, the epidemic of 1994–5 appeared to be centred near the city of Mykolajiv, with over 70% of cases occurring in the Mykolajivska oblast. The epidemic appeared to begin in Ruspublika Krim, possibly due to the breakdown of the integrity of the water supply, and spread rapidly to the Mykolajivska oblast. The shape of the epidemic curve (Fig. 3) in 1995 was completely different than that of 1994, and suggests a continuation of the 1994 epidemic once temperatures became warmer. Fatalities due to cholera occurred throughout the epidemic, and likely resulted from the prior condition of the patient rather than the quality or availability of medical care.

The number of cases of cholera reported during the 1994–5 Ukrainian epidemic was likely an underestimate for several reasons. Cholera only came to the

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attention of the hospitals and health authorities when patients contacted doctors for treatment. In Ukraine antibiotics are widely available without prescription in local markets. Furthermore, hospitals were not in optimal operating condition during the epidemic, and it is thought that many individuals showing milder symptoms, especially among the homeless, may have chosen not to contact doctors. The potential for the use of oral rehydration therapy outside of the hospital setting is unknown. The total number of cases during the epidemic may therefore have been an order of magnitude greater than was reported to the health system. Though isolation of *V. cholerae* was required for a positive diagnosis of cholera, the symptoms of cholera were highly characteristic and there did not appear to be any cases of clinical manifestation of the disease reported to doctors where the organism was not isolated. It was estimated that failure to isolate the organism would have caused a missed diagnosis in only about 10% of cases. Carriers were identified throughout the epidemic with the exception of the first 2 weeks in 1994, when the nature and extent of the epidemic was first becoming apparent. The identification of carriers was necessarily limited by the available resources, and probably underestimated the true magnitude of carriage of *V. cholerae* in the population as a whole.

V. cholerae have been isolated frequently from ponds, sea water and river water in various countries [8], and have been found in Ukrainian aquatic environments during the period 1970–95, though the proportion of toxigenic strains has declined between 1970 and the present [7]. While acquisition of the organism from aquatic sources has previously been found to cause both sporadic and epidemic cholera [8], the explosive increase in cholera cases in 1994 and 1995 and equally sudden disappearance of cholera in 1996 argue for additional mechanisms causing cholera transmission and/or susceptibility. It is possible that introduction of a new strain of *V. cholerae* into coastal waters may have precipitated the epidemic in the same way that contaminated ship's ballast water has been proposed to have caused the South American cholera epidemic [27].

Though the origin of the epidemic is not known, data indicating that toxigenic *V. cholerae* was present in the environment before the epidemic of 1994–5 suggest that environmental reservoirs may have been involved. First, an epidemiological correlation between onset of disease and consumption of fish was made for 51% of the cases with known or suspected mechanisms of transmission from Mykolajiv during the height of the epidemic. A 1993 fish isolate from a lake in Odeska oblast, 95-0745, appeared to be identical or similar to isolates from patients during the outbreak. Shellfish, crustaceans and finfish have been previously implicated as source of bacteria for cholera outbreaks, and *V. cholerae* O1 has been found in shellfish and on the skin and in intestines of finfish [9–11]. The rise in carriers in Odessa in 1993 (Table 1) may also have marked the beginning of the epidemic, since it came at the end of a period of very low prevalence of *V. cholerae* and immediately preceded the epidemic. Secondly, the fact that *V. cholerae* typical of the epidemic strains were found in sea water, sewage and river water suggests that during the epidemic contamination of the water supply was extensive. No *V. cholerae* were isolated from the drinking water supply, indicating that water treatment processes were sufficient to render drinking water safe. Though it is impossible to determine from the available data whether the human or environmental sources of *V. cholerae* precipitated the epidemic, it should be noted that in 1991 *V. cholerae* O1 was detected in water and plankton specimens collected in the Black sea near Ukraine 4 weeks prior to a cholera outbreak in the same area [28, 29]. These data suggest that monitoring of the environment for toxigenic *V. cholerae* may help predict future epidemics or outbreaks.

All disease isolates and most environmental isolates assessed microbiologically in this study were potentially virulent in that they produced CT and carried the *ctx* gene encoding CT. The zonula occludens toxin gene (*zot*) does not occur independently of cholera toxin gene (*ctx*) in clinical isolates of *V. cholerae* [30–32], though rare combinations containing natural deletions of either *zot*, *ctx* and/or *ace* genes have been found [33] and the *ctx* gene appears to be frequently deleted from environmental *V. cholerae* O1 strains [33]. Since it is likely that other toxin genes in the virulence cassette (*zot* and *ace*) would be present in all *ctx*-positive human and environmental *V. cholerae* isolates, the presence of these genes was not assessed in this study.

All *V. cholerae* isolates involved in the 1994–5 Ukrainian epidemic were resistant to multiple antibiotics. The routine use of antibiotics early in the epidemic for treatment of patients and carriers may have contributed to the selection of antibiotic resistant variants of *V. cholerae*. However, the isolation from a fish in 1993 of *V. cholerae* with an antibiogram similar

to that of many epidemic strains argues that resistant strains may have been present in the environment before the epidemic began.

The patterns of resistance in the Ukrainian isolates were the same or very similar to a representative seventh pandemic *V. cholerae* El Tor O1 isolate from Pakistan. In a survey of *V. cholerae* strains from around the world, Sciortino and colleagues [34] found that 16·5% of strains were resistant to ampicillin, 12·7% to trimethoprim/sulphamethoxazole, and very low percentages resistant to other antibiotics in Vitek antibiotic screening assays. Indian *V. cholerae* strains were resistant to several antibiotics, especially streptomycin, furazolidone and co-trimoxazole, though resistance to ampicillin, chloramphenicol and nalidixic acid also increased dramatically in the period between 1992 and 1994 [35]. Multiple resistance has also been described in *V. cholerae* O1 isolates from an outbreak in Nigeria [36]. In this case, resistance to ampicillin, penicillin, streptomycin and tetracycline predominated, while few isolates were resistant to sulphamethoxazole/trimethoprim. There also appeared to be much greater variability in resistance patterns than was seen with the Ukrainian isolates. Plasmid-borne resistance to several antibiotics, including ampicillin, kanamycin, streptomycin, sulphonamide, tetracycline, chloramphenicol, spectinomycin and trimethoprim, was found in *V. cholerae* O1 strains involved in a major epidemic in 1985–6 in the Horn of Africa [37], while *V. cholerae* O1 El Tor Ogawa isolates from Uganda contained a 130 Mda plasmid that encoded resistance to ampicillin, streptomycin, tetracycline, chloramphenicol, trimethoprim and sulphonamide [38]. Multiple antibiotic resistance was found in isolates from a 1991 cholera epidemic in Ecuador, consisting of resistance to chloramphenicol, doxycycline, kanamycin, streptomycin, sulphisoxazole, tetracycline and trimethoprim/sulphamethoxazole [11]. This pattern of resistance is different from any resistance pattern found in the Ukrainian isolates and also differs from the Peruvian strain used as a control (94-0457).

The patterns of antibiotic resistance seen in *V. cholerae* O1 in Ukraine are therefore clearly different from patterns of antibiotic resistance seen in this organism in parts of the world other than India. Resistance patterns of these Ukraine isolates are, however, similar to the antibiotic resistance encoded by a conjugative transposon in *V. cholerae* O139, which encodes resistance to sulphamethoxazole, trimethoprim and streptomycin [22]. A related element from El Tor strains in India may be widely disseminated in *V. cholerae*. The co-transfer of trimethoprim, sulphamethoxazole and streptomycin resistance from *V. cholerae* to an *E. coli* recipient in the absence of detectable plasmids, demonstrated here, provides genetic evidence that multiple antibiotic resistance determinants may be located on a conjugative transposon in Ukrainian isolates.

There was occasional difficulty in interpretation of intermediate resistance results, especially for tetracycline. Almost all isolates appeared to have intermediate resistance to tetracycline when the disk diffusion test was used, whereas only three exhibited intermediate resistance to tetracycline when the Vitek system was used. All were sensitive in the agar dilution assay, which was used in an attempt to reconcile conflicting data. Difficulty in obtaining consistent results for tetracycline when using the Vitek system to evaluate the antibiotic resistance of members of the *Enterobacteriaceae* has been noted previously [39], though the large numbers of intermediate resistance results found with the disk diffusion assays remains unexplained. The control strains used gave results within the acceptable range for all methods used. These findings underscore the need for antibiotic resistance methods and interpretive criteria specifically for *V. cholerae* to avoid continued reliance on those used for the *Enterobacteriaceae*.

Together, the phenotypic characteristics and antibiotic resistance patterns of *V. cholerae* isolates from the Ukraine epidemic suggest that a relatively homogenous group of strains was involved in the epidemic. The similarity of these isolates compared with a patient isolate from Pakistan (93-0608) further suggests these isolates are closely related to other seventh pandemic isolates from Asia. In addition, the pattern of antibiotic resistance is remarkably similar to that of *V. cholerae* isolates carrying a conjugative transposon encoding multiple antibiotic resistances. Characterization of both patient and environmental isolates described here using molecular biological techniques is required to further elucidate relationships between strains, and is the subject of a separate communication.

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REFERENCES

1. Kaper JB, Morris JG Jr, Levine MM. Cholera. Clin. Microbiol Rev 1995; **8**: 48–86.
2. Popovic T, Olsvik Ø, Blake PA, Wachsmuth K. Cholera in the Americas: foodborne aspects. J Food Protect 1993; **56**: 811–21.
3. Tauxe RV, Mintz ED, Quick RE. Epidemic cholera in the new world: translating field epidemiology into new prevention strategies. Emerg Infect Dis 1995; **1**: 141–6.
4. Nair GB, Albert MJ, Shimada T, Takeda Y. *Vibrio cholerae* O139 Bengal: the new serogroup causing cholera. Rev Med Microbiol 1996; **7**: 43–51.
5. Begue RE, Meza R, Castellares G, et al. Outbreak of diarrhea due to *Vibrio parahemolyticus* among military personnel in Lima, Peru. Clin Infect Dis 1995; **21**: 1513–4.
6. World Health Organization. Cholera in 1995. Weekly Epidemiol Rec 1996; **71**: 157–64.
7. Narkevich MI, Onischenko GG, Lomov JM, Moskvitina EA, Podosinnikova LS, Medinsky GM. The seventh pandemic of cholera in the USSR, 1961–89. Bull WHO 1993; **71**: 189–96.
8. Islam MS, Drasar BS, Sack RB. The aquatic environment as a reservoir of *Vibrio cholerae*: A review. J Diarrhoeal Dis Res 1993; **11**: 197–206.
9. Kaysner CA, Hill WE. Toxigenic *Vibrio cholerae* O1 in food and water. In: Wachsmuth IK, Blake PA, Olsvik Ø, eds. *Vibrio cholerae* and cholera: molecular to global perspective. Washington, DC: ASM Press, 1994: 27–39.
10. Mintz ED, Popovic T, Blake PA. Transmission of *Vibrio cholera* O1. In: Wachsmuth IK, Blake PA, Olsvik Ø, eds. *Vibrio cholerae* and cholera: molecular to global perspective. Washington, DC: ASM Press, 1994: 345–56.
11. Weber JT, Mintz ED, Cañizares R, et al. Epidemic cholera in Ecuador: multi-drug resistance and transmission by water and seafood. Epidemiol Infect 1994; **112**: 1–11.
12. Honma Y, Higa N, Tsuji T, Iwanaga M. Comparison of a reversed passive latex agglutination and a polymerase chain reaction for identification of cholera toxin producing *Vibrio cholerae* O1. Microbiol Immunol 1995; **39**: 59–61.
13. Iwanaga M, Yamamoto K, Higa N, Ichinose Y, Nakasone N, Tanabe M. Culture condition for stimulating cholera toxin production by *Vibrio cholerae* O1 El Tor. Microbiol Immunol 1986; **30**: 1075–83.
14. Fields, PI, Popovic T, Wachsmuth K, Olsvik Ø. Use of polymerase chain reaction for detection of toxigenic *Vibrio cholerae* O1 strains from the Latin American cholera epidemic. J Clin Microbiol 1992; **30**: 2118–21.
15. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests; approved standard, 5th ed. Vol. 13, no. 24. Villanova, PA: NCCLS, 1993; M2-A5.
16. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 3rd ed. Vol. 13, no. 25. Villanova, PA: NCCLS, 1993: M7-A3.
17. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests: sixth informational supplement. Vol. 15, no. 14. Villanova, PA: NCCLS, 1995: M100-S6.
18. Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 1979; **1**: 1513–23.
19. Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 1981; **145**: 1365–73.
20. Eckhardt T. A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. Plasmid 1978; **1**: 584–8.
21. Barton BM, Harding GP, Zuccarelli AJ. A general method for detecting and sizing large plasmids. Anal Biochem 1995; **226**: 235–40.
22. Waldor MK, Tschäpe H, Mekalanos JJ. A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. J Bacteriol 1996; **178**: 4157–65.
23. Narkevich MI, Onischenko GG, Lomov Yu.M, Kuregjan AA, Medinskij GM, Moskvitina EA. The practice of cholera epidemiological surveillance in the Soviet Union in the period of the seventh pandemic. J Med Epidemiol Immunol 1991; **6**: 40–3.
24. Cholera is a miasmatic disease. The bulletin of public hygiene, criminal and practical medicine, St. Petersburg 1907; VII. Typography of the Ministry of Internal Affairs. (Title translated from Russian by A. N. Kravetz.)
25. Onickanskij MS. About the spread of cholera in Russia. The bulletin of public hygiene, criminal and practical medicine, St. Petersburg 1911; XI: 355-7, 667-97, 803-35. Typography of the Ministry of Internal Affairs. (Title translated from Russian by A. N. Kravetz.)
26. Uljanova LD. The periodicity in development of cholera epidemics. Profilakticheskaja medicina 1922; **7–8**: 80–8. (Title translated from Russian by A. N. Kravetz.)
27. Tauxe R, Seminario L, Tapia R, Libel M. The Latin American epidemic. In: I. K. Wachsmuth, P. A. Blake, Ø. Olsvik, eds. *Vibrio cholerae* and cholera: molecular to global perspective. Washington, DC: ASM Press, 1994: 321–44.

28. Huq A, Hasan AK, Losonsky G, Colwell RR. Occurrence of toxigenic *V. cholerae* O1 and *V. cholerae* non-O1 in professional divers and dive sites in the United States, Ukraine, and Russia. FEMS Microbiol Letters 1994; **120**: 137–42.
29. Colwell RR, Huq A. Environmental reservoir of *Vibrio cholerae*: the causative agent of cholera. Ann New York Academy Sci 1994; **740**: 44–54.
30. Faruque SM, Comstock L, Kaper JB, Albert MJ. Distribution of *Zonula occludens* toxin (*zot*) gene among clinical isolates of *Vibrio cholerae* O1 from Bangladesh and Africa. J Diarrhoeal Dis Res 1994; **12**: 222–4.
31. Johnson JA, Morris JG Jr, Kaper JB. Gene encoding zonula occludens toxin (*zot*) does not occur independently from cholera enterotoxin genes (*ctx*) in *Vibrio cholerae*. J Clin Microbiol 1993; **31**: 732–3.
32. Karasawa T, Mihara T, Kurazono H, et al. Distribution of the *zot* (zona occludens toxin) gene among strains of *Vibrio cholerae* O1 and non-O1. FEMS Microbiol Lett 1993; **106**: 143–6.
33. Kurazono H, Pal A, Bag PK, et al. Distribution of genes encoding cholera toxin, zonula occludens toxin, accessory cholera toxin, and El Tor hemolysin in *Vibrio cholerae* of diverse origins. Microb Pathog 1995; **18**: 231–5.
34. Sciortino CV, Johnson JA, Hamad A. Vitek system antimicrobial susceptibility testing of O1, O139, and non-O1 *Vibrio cholerae*. J Clin Microbiol 1996; **34**: 897–900.
35. Mukhopadhyay AK, Garg S, Nair GB, et al. Biotype traits and antibiotic susceptibility of *Vibrio cholerae* serogroup O1 before, during and after the emergence of the O139 serogroup. Epidemiol Infect 1995; **115**: 427–34.
36. Olukoya DK, Ogunjimi AA, Abaelu AM. Plasmid profiles and antimicrobial susceptibility patterns of *Vibrio cholerae* O1 strain isolated during a recent outbreak in Nigeria. J Diarrhoeal Dis Res 1995; **13**: 118–21.
37. Coppo A, Columbo M, Pazzani C, et al. *Vibrio cholerae* in the horn of Africa: epidemiology, plasmids, tetracycline resistance gene amplification, and comparison between O1 and non-O1 strains. Am J Trop Med Hyg 1995; **53**: 352–9.
38. Kruse H, Sørum H, Tenover FC, Olsvik Ø. A transferable multiple drug resistance plasmid from *Vibrio cholerae* O1. Microb Drug Rest 1995; **1**: 203–10.
39. Berke I, Tierno PM Jr. Comparison of efficacy and cost-effectiveness of BIOMIC VIDEO and Vitek antimicrobial susceptibility test systems for use in the clinical laboratory. J Clin Microbiol 1996; **34**: 1980–4.