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Changes in *Vibrio cholerae* O1 strains isolated in Romania during 1977–95

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SUMMARY

Six hundred and twenty-four *Vibrio cholerae* O1 strains, 623 serotype Ogawa and one serotype Inaba, isolated in Romania between 1977–95 were tested to detect all changing traits concerning serogroup, serotype, biotype, phage type and resistotype patterns and subsequently, the possible epidemiological relationship among these strains. Biotyping revealed one classical, 580 eltor strains and 43 intermediary variants. When tested with Mukerjee phages, 546 (87%) strains were sensitive and 78 (13%) resistant. One phage type (M4) dominated during 1977–90, two phage types (M4 and M5) exhibited the same high frequencies during 1991, a diversity of types occurred during 1993–4 whereas in 1995, two phage types (M4 and M5) showed similar distributions again. Five patterns of drug susceptibility were successively described during 1977–95. The most prominent changes in *Vibrio cholerae* O1 strains were noticed during 1993–4: the highest number of non-typable strains and intermediary variants, the widest spectrum of phage types and of multidrug resistance. In 1995, the strains reverted to the previous typable forms but a new drug resistance pattern was noticed.

INTRODUCTION

The seventh pandemic of cholera due to *Vibrio cholerae* group O1 biotype eltor (1) strains shows no sign of declining; on the contrary, since 1971 European countries [2–4] as well as parts of Africa [5, 6], Asia [7, 8], Australia [9], Latin [10] and Central [11] America have been affected. A new strain of *Vibrio cholerae* group O139 which was isolated in several parts of India, in October 1992, spread to the entire Indian subcontinent and several countries in Asia during 1993 [12]. In recent years, researchers reported several changing traits of *Vibrio cholerae* O1 strains isolated in different parts of the world; atypical biotyping results [13] and of multiple drug resistance [14]. These results emphasised an evident mobility in genetic elements accounting for the traits of *Vibrio cholerae* O1 strains evolving over years and a possible connection to the upsurge of *Vibrio cholerae* O139 [13]. During the past 20 years cholera made many appearances in Romania, the highest frequency being reported in the Danube Delta and its neighbouring districts; the occurrence in other areas of the country has often been linked to the Danube Delta (cases with a history of travelling, delta fish and water consumption) as well as to imported cases.

In the present studies, 624 *Vibrio cholerae* O1 strains isolated in Romania during 1977–95 were studied with the purpose of monitoring all changing traits of serogroup, serotype, biotype, phage type and drug resistance patterns of the cholera agent and especially in the last few years, when a large number of migrant workers returned home from Asian endemic areas.

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MATERIALS AND METHODS

Strain selection

A total of 624 *Vibrio cholerae* O1 strains (553 from patients with acute watery diarrhoea, 42 from contacts, 29 from water samples) have been studied. The strains were isolated in Romania, during the outbreaks of 1977, 1987, 1990, 1993, 1994 and 1995. The strains isolated on Bile Salt Agar medium were tested with polyvalent O1 and monospecific Ogawa and Inaba antisera (prepared on rabbits).

Biochemical identification

A battery of standard tests was done [15]; oxidase, acid production from D-glucose, lactose, sucrose, D-mannitol, mannose, L-arabinose, inositol, adonitol, indole, malonate, lysin decarboxylase, ornithine decarboxylase and arginine dehydrolase.

Biotyping

Five biotyping tests were used: bacteriophage susceptibility (to eltor 5 and classical IV phages), haemagglutination direct test (with chicken and group 0 human red cells), Voges Proskauer test, haemolysis (5% sheep blood agar plates) and polymyxin B (50 U) susceptibility [15].

Bacteriophage typing

It was performed with the Mukerjee set of cholera phages (5 eltor and 4 classical phages) [16–18]. The propagation and storage of these phages are routinely performed in our laboratory. The phage preparations were used at the routine test dilutions (RTDs) controlled on standard *Vibrio cholerae* O1 classical number 157 and eltor MAK 757 strains. The isolates to be tested were grown overnight in broth cultures. From the overnight growth, a nutrient broth was inoculated and incubated 2 h at 37 °C. A lawn of bacteria was then seeded on to the surface of a nutrient agar plate 0·5%. A drop of the phage diluted to RTD was applied to the bacterial lawn. The plates were incubated overnight at 37 °C and the plaques were read the next day.

Susceptibility to antimicrobial agents

Susceptibility of *Vibrio cholerae* O1 strains to a variety of antimicrobial agents was performed by the disk diffusion technique [15] with commercial disks (Oxoid).

Recording of results

The following criteria were used to record the results: bacteriophage susceptibility was considered the most important, the strains sensitive to eltor 5 or classical IV phage being recorded as typable; the strains resistant to these phages were recorded as non-typable and had to present at least four other positive tests in order to be included into the corresponding biotype. When referring to the strain phage typing by Mukerjee's set of phages, the strains sensitive/resistant to eltor 1–5 phages were reported as sensitive/resistant.

RESULTS

All 624 strains agglutinated with polyvalent O1 antiserum; 623 strains belonged to serotype Ogawa and one strain to serotype Inaba. All strains belonged to group Heiberg I.

Bacteriophage susceptibility: out of 624 *Vibrio cholerae* O1 strains, 508 proved to be sensitive to eltor 5 phage but 8 strains were simultaneously sensitive to classical IV phage. Out of the 116 remaining strains resistant to eltor 5 phage, one strain was sensitive to classical IV.

Haemagglutination (direct) test: when performed simultaneously with chicken and group O human red cells, 26 strains exhibited double negative reactions and 94 double positive in both cases; the remaining 504 strains exhibited haemagglutination only with chicken erythrocytes as follows: 95% of the strains isolated in 1987, 71% in 1993, 77% in 1994 and 89·3% in 1995.

Voges Proskauer test: 57% of strains were positive.

Haemolysis test (plate method). This test indicated that the haemolytic property of *Vibrio cholerae* O1 strains isolated in our geographical area during 1977–95 differed from very low (4·7%) level in strains isolated in 1977, showing an increase to 56% in 1990, 93% in 1993, 98% in 1994 and 100% in 1995.

Polymyxin B (50 U) susceptibility. Any zone of inhibition was interpreted as a positive result [15]. The *Vibrio cholerae* O1 in very high percentage (88–100%) but an increasing trend of polymyxin B susceptibility of strains from 17% in 1993 to 78% in 1995 was observed.

Phage typing aspects. Out of 624 *Vibrio cholerae* O1 strains/tested with the Mukerjee set of phages, 546

Table 1. *Lysosensitivity aspects in* Vibrio cholerae *O1 strains isolated in Romania during 1977–95*

	Basu-Mukerjee phages (eltor)						
						Sensitive strains	
Lysotypes	e_1	e_2	e_3	e_4	e_5	Number	Per cent
Mukerjee's							
M_1	+	+	+	+	+	10	1·6
M_2	+	+	+	−	+	—	—
M_3	+	+	−	+	+	—	—
M_4	+	+	−	−	+	322	51
M_5	+	−	−	−	+	75	12
M_6	−	+	−	−	+	59	9·4
Chattopadhyay's and colleagues							
C_a	−	−	−	−	+	20	3·2
C_b	−	+	−	−	−	14	2·2
C_c	−	+	+	−	+	2	0·32
C_d	+	−	−	−	−	28	4·4
C_e	+	−	+	−	+	2	0·32
C_f	+	+	−	−	−	14	2·2
Total number of lysosensitive strains						546	87
Total number of lysoresistant strains						78	13
Total number of *V. cholerae* O1 strains						624	—

(87%) strains were sensitive and 78 (13%) resistant. These 546 sensitive strains have been divided into 10 phage types of which 4 types (M4, M6, M5 and M1) described by Mukerjee [16] and 6 types (Ca→Cf) (Table 1) described only later by Chattopadhyay and colleagues [19] when using the same Mukerjee set of phages. The most common was the M4 (51% strains) followed by M5 (12% strains), M6 (9·4%), Ca (3·2%), Cd (4·4%), Cb and Cf (both 2·2%) and Cc and Ce (both 0·32%) (Table 1). In point of distribution, phage type M4 was predominant during 1977–90; M4 and M6 showed almost the same frequency in 1991, an evident expanding of several phage types was recorded during 1993–4, whereas M4 and M5 exhibited the same high frequency in 1995. Out of 78 resistant strains, the highest percentage of 70% was isolated in 1993 followed by 26% in 1994 (many being isolated from imported cases).

Susceptibility to antimicrobial agents. Drug susceptibility of *Vibrio cholerae* O1 strains showed interesting variations (Table 2) which could be divided into three groups: strains isolated during 1977–87 exhibiting resistance to polymyxin B (100%–88·6%) only; strains isolated in 1990 and 1991 exhibiting resistance to polymyxin B (91–100%) and furazolidone (80–100%). After 1991, the polymyxin B resistance of *Vibrio cholerae* O1 strains showed a decreasing trend. In 1991 although in a small number (9·8%) the first *Vibrio cholerae* O1 strains resistant to tetracycline were reported; strains isolated during 1993–5 with increasing resistance as follows: (*a*) the strains of 1993 with resistance markers (polymyxin B, furazolidone, co-trimoxazole and tetracycline) more than 83% frequency; (*b*) the strains of 1994 with the same predominant markers (Table 3), to which a new one (to vibriostatic agent O/129) was added; (*c*) the strains of 1995 with constantly three old markers (furazolidone, co-trimoxazole and O/129) to which a new one (to nalidixic acid) was added; polymyxin B resistance was present only in 22% of the strains, whereas tetracycline resistance has almost vanished (1·8%).

DISCUSSION

Out of 624 *Vibrio cholerae* O1 strains, 623 belonged to serotype Ogawa and one to serotype Inaba, the last one imported from Pakistan in 1991. A total of 553 strains were isolated from ill subjects (15 children and 538 adults, ranging in age from 40–77 years with 3 terminal cases); 42 strains were isolated from contacts (with asymptomatic infections) ranging in age from 20 to 30 years and 29 strains isolated from water samples (Danube surface water, water containers, sewage water, leaky pipelines, fountain water). The highest number of strains 401 (64%) originated in the Danube

Table 2. *Drug resistance of* Vibrio cholerae *O1 strains isolated between 1977–95*

Year of isolation	Number of tested strains	Drug resistance (%)							
		Polymyxin B 50 U	Furazolidone 100 mcg	Co-trimoxazole 25 mcg	Tetracycline 30 mcg	Chloramphenicol 30 mcg	Doxycycline 30 mcg	O/129 150 mcg	Nalidixic acid 30 mcg
1977	10	100	0	0	0	0	0	0	0
1987	67	88·6	0	0	0	0	0	0	0
1990	164	91·6	80	5	0	0	0	0	1·6
1991	105	100	100	17	9·8	9	20·6	19	5·2
1993	24	83	100	100	95	17	20·8	4	20·8
1994	138	74	100	100	100	63	29	100	0
1995	116	22	100	100	1·8	3	0	100	97

Table 3. *Predominant traits of* Vibrio cholerae *O1 strains isolated in Romania between 1977–95*

Year of isolation	Number of strains	Number of typable strains	Number of non typable strains	Intermediary variants	Lysotypes			Drug resistance*					
					High frequent	Frequent	Sporadic						
1977	10	9	1 (10%)	—	M_4	—	M_1, M_5, C_a	Poly B					
1987	67	65	2 (2·9%)	—	M_4	—	C_f	Poly B					
1990	164	147	17 (10%)	2 (1·2%)	M_4	—	M_5, C_f, M_1	Poly B	F				
1991	105	102	3 (2·8%)	1 (0·9%)	M_4/M_6	—	C_f	Poly B	F				
1993	24	7	17 (70%)	7 (2·9%)	—	M_5, C_a	M_4, C_c, C_f	Poly B	F	Co	T		
1994	138	65	73 (52%)	33 (23%)	—	M_4, M_5, C_d	C_a, C_b C_e, C_f	Poly B	F	Co	T	O/129	
1995	116	114	2 (1·7%)	—	M_4/M_5	—	M_6	—	F	Co	—	O/129	Nalidixic acid
Total no. of strains	624	509	115	43									

* Drug resistance: Poly B, polymyxin B (50 U); F, furazolidone; Co, co-trimoxazole; T, tetracycline; O/129, vibriostatic agent.

Delta and the neighbouring districts, while 223 (36%) strains were isolated in the central areas. In Romania, the season favouring cholera lasted from July to the end of October (warm season). In the Danube Delta, cholera transmission was probably linked to: drinking of surface water directly from the Danube (49%); water consumption from infected storage containers, ubiquitous in households; food (fish) handling with dirty hands (27%); unfiltered and unchlorinated water through leaky pipes. In other areas, occurrence of cholera was often linked to: history of travelling in the Danube Delta area or delta fish consumption; water consumption from fountains infected with cholera vibrios; cholera import by migrant workers returning home from Asia endemic areas (2 cases from Pakistan in 1991 and 1992, 21 cases from Turkey in 1993–4 and 1 case from Turkey in 1995).

When considering the susceptibility to eltor 5 and classical IV phages as the most important aspect of biotype differentiation, out of 624 strains, 508 proved to be sensitive and 116 resistant to eltor 5. However, in the first stage of this analysis, out of these 508 strains only 500 were classified as belonging to biotype eltor while 8 strains (1 isolated in 1987 and 7 in 1990) proved to be simultaneously sensitive to classical IV phage. Moreover, out of the 116 strains resistant to eltor 5 phage, 1 strain (isolated in 1990) proved to be sensitive to classical IV phage.

In the second stage of our analysis, when considering the results presented by the other four biotyping tests, we noticed: the 8 strains simultaneously sensitive to eltor 5 and classical IV phages constantly exhibited four tests corresponding to biotype eltor; the unique strain resistant to eltor 5 phage but sensitive to classical IV exhibited four tests corresponding to biotype eltor; out of the remaining 115 non-typable strains (simultaneously resistant to eltor 5 and classical IV phages), 72 strains have been included in biotype eltor based upon four other biotyping tests whereas 43 strains (10 negative and 33 positive with both erythrocyte species) have been stated as intermediary variants. Similar aspects were reported by other authors [10]. The highest percentage of intermediary variants as well as that of non-typable strains were noticed during 1993 (29% and 70%) followed by 1994 (23% and 52%) (Table 3), a great number of these strains being isolated in patients coming from abroad.

When considering the phage sensitivity aspects determined by the Mukerjee's set of phages, several conclusions were drawn: phage type M4 predominant in our area during 1977–91 and exhibiting a new increasing trend in 1995 is the ubiquitary predominant phage type of Asian origin mentioned by Chattopadhyay and colleagues [19]; phage types M6 and M5 reported by the same authors as 'almost vanished' from the Indian territory were frequently reported in our country in the epidemics of 1991 and 1995. This could be interpreted as a re-emergence of these two phage types in our geographical area; the greatest variety of phage types, as well as the highest number of non-typable strains recorded during 1993–4, could be linked with the greatest variety of phage types, as well as the greatest number of imported cholera cases (migrant workers coming from Asia). Simultaneously with the changing in phage sensitivity, an evident change of the drug resistance pattern was noticed in isolates of 1993–5 (Table 3).

During 1994–5, 100% cholera strains became resistant to vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine (O/129) (150 μg). Considering this aspect, the taxonomical usefulness of the test with vibriostatic agent in order to differentiate between *Vibrio* and *Aeromonas* genera is no longer valid. That was previously mentioned also by other authors [20]. The resistance to vibriostatic agent was constantly linked with co-trimoxazole and furazolidone resistance; linked resistance to O/129 and co-trimoxazole in *Vibrio cholerae* O1 strains has been also previously reported [13, 20]. The emergence of a very high resistance to nalidixic acid (93%) in the Romanian strains isolated in 1995 must be mentioned and could be related to the widespread use of this drug before, in the treatment of shigella and tetracycline resistant cholera cases. The loss of polymyxin B (50 U) resistance in a very large number of eltor strains (78%) recorded during 1995, made the polymyxin B test no longer valid in the biotyping of *Vibrio cholerae* O1 strains.

In previous studies, the authors reported that 20% of the Romanian strains isolated during 1977–94, exhibited an incomplete 'virulence cassette' (unpublished results), these strains were distributed into 18 ribotypes exhibiting a genetic divergence ranging between 0·05 and 0·48. The ribotype B21 was the most frequent (unpublished results); it was not an indigenous but an endemic ribotype also described by other authors [21] in different parts of the world. A new ribotype B27 was found in all tested strains isolated in Romania in 1995 [22].

Out of 624 *Vibrio cholerae* O1 strains isolated during 1977–95, 580 belonged to the biotype eltor, 1 strain to

classical and 43 strains were considered intermediary variants.

The most proeminent changes (high number of non-typable strains and intermediary variants, diversity of phage types and multiple drug resistance) were recorded for the first time in strains isolated in 1993, as the emergence of *Vibrio cholerae* O139 was recorded in other parts of the world. These changes lasted for 2 years (1993–4). In 1995, the strains reverted to phage typable forms with only 2 predominant phage types and a new drug resistance pattern. The Romanian *Vibrio cholerae* O1 strains isolated during 1993–5 exhibited the largest tendency for the mutation and rearrangements of genetic elements among which the phages proved to play a very important role.

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