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Isolation frequency and susceptibility pattern of non-O1 and non-O139 Vibrio cholerae in a tertiary health care laboratory, 1999-2012

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Isolation frequency and susceptibility pattern of non-O1 and non-O139 *Vibrio cholerae* in a tertiary health care laboratory, 1999–2012

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تردد عزل أنماط حساسية ضمات الكوليرا الـلا O1 والـلا O139 في أحد مختبرات الرعاية الصحية الثالثية، في الفترة ما بين 1999-2012

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الخلاصة: لقد سُلط الضوء – عالمياً – في العقد الماضي على أهمية سلالات ضمات الكوليرا الـلا O1 والـلا O139. وقد هدفت هذه الدراسة إلى تقييم تكرار عزل الكوليرا الـلا O1 والـلا O139 وأنماط حساسيتها للأدوية في باكستان. فأجري تحليل استعادي لبيانات عينات البراز التي حدث فيها نمو لضمات الكوليرا الـلا O1 والـلا O139 والتي عُزلت في مختبر إحالات وطني من عام 1999 إلى عام 2012، وقيِّمت مقاومتها للأمبيسيلين والتراسيكلين والكلورامفينيكول والكوتريموكسازول والأوفلوكساسين. لقد أنتجت عينات البراز المقدَّمة خلال الأعوام 1999-2012 والتي بلغ مجموعها 95800 عينة ما مجموعه 3668 سلالة من سلالات ضمة الكوليرا، كان 6٪ منها ضمات كوليرا لا O1 ولا O139. ووجد أن معدل العزل كان مرتفعاً في فصل الصيف، وبلغ ذروته في عام 2003. وأظهرت بيانات حساسية الميكروبات للأدوية زيادة في مقاومة هذه الذراري للكوتريموكسازول والأمبيسيلين، مع بقائها حساسة للأوفلوكساسين. إنه من الضروري القيام بترصد نشط للأنماط المصلية ولحساسيتها للأدوية، من أجل التنبؤ بالأوبئة المستقبلية وتحديد التدابير اللازمة للحد من المرض.

ABSTRACT In the past decade the importance of non-O1 and non-O139 strains of *Vibrio cholerae* has been highlighted globally. This study aimed to evaluate the frequency and antimicrobial susceptibility profile of non-O1 and non-O139 *V. cholerae* in Pakistan. Data of stool specimens yielding growth of non-O1 and non-O139 *V. cholerae* isolated at a national referral laboratory from 1999 to 2012 were retrospectively analysed and evaluated for resistance to ampicillin, tetracycline, chloramphenicol, co-trimoxazole and ofloxacin. A total of 95 800 stool samples submitted over 1999–2012 yielded 3668 strains of *V. cholerae*, of which 6% were non-O1 and non-O139 *V. cholerae*. A high isolation rate was found in the summer season, with a peak in the year 2003. Antimicrobial susceptibility data revealed increasing resistance to co-trimoxazole and ampicillin, but strains remained highly susceptible to ofloxacin. Active surveillance of serotypes and antimicrobial susceptibility is essential to predict future epidemics and define measures to curtail the disease.

Fréquence d'isolation et profil de sensibilité de *Vibrio cholerae* non-O1 et non-O139 dans un laboratoire de soins de santé tertiaires, 1999-2012

RÉSUMÉ Au cours des dix dernières années, l'importance des souches de *Vibrio cholerae* non-O1 et non-O139 a été mise en avant à l'échelle mondiale. La présente étude visait à évaluer la fréquence de l'isolation des souches de *Vibrio cholerae* non-O1 et non-O139 et leur profil de sensibilité aux antimicrobiens au Pakistan. Les données d'échantillons de selles ayant permis la croissance de *V. cholerae* non-O1 et non-O139 isolés dans un laboratoire national spécialisé entre 1999 et 2012 ont été analysées et évaluées rétrospectivement pour leur résistance à l'ampicilline, la tétracycline, au chloramphénicol, au co-trimoxazole et à l'ofloxacine. Au total, 95 800 échantillons de selles soumis entre 1999 et 2012 ont produit 3668 souches de *V. cholerae*, parmi lesquelles 6 % étaient des souches de *V. cholerae* non-O1 et non-O139. Un taux d'isolation élevé a été observé au cours de l'été, avec un pic en 2003. Les données relatives à la sensibilité aux antimicrobiens ont révélé une augmentation de la résistance au co-trimoxazole et à l'ampicilline, mais les souches restaient fortement sensibles à l'ofloxacine. Une surveillance active des sérotypes et de la sensibilité aux antimicrobiens est essentielle pour prévenir les épidémies et définir des mesures permettant d'enrayer la maladie.

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Introduction

Food- and water-borne illnesses are leading public health problems around the world. Although in developed countries the predominant bacterial causes of diarrhoea include pathogens such as *Salmonella* and *Campylobacter* spp. (*1*), in developing countries *Vibrio cholerae* still remains the top cause of watery diarrhoea (*2*). *V. cholerae* is classified to over 200 serotypes on the basis of variations in the lipopolysaccharide somatic antigen (*3*). *V. cholerae* belonging to serogroups O1 and O139 has been associated with epidemics and pandemics of cholera due to production of cholera toxin (*2–5*). Other serotypes are collectively known as non-O1 and non-O139 or non-typeable *V. cholerae*. A majority of these strains are usually responsible for sporadic cases of diarrhoea (*6–8*). However, the pathogenic potential of non-O1 and non-O139 *V. cholerae* should not be underestimated as some strains are clearly pathogenic, due either to cholera toxin production or to other virulence factors such as heat-stable enterotoxin, haemolysin, repeats-in-toxin and type 3 secretion systems (*7*). Occasionally, these strains are responsible for outbreaks of cholera (*9–11*). According to the United States Centers for Disease Control and Prevention (CDC), non-O1 and non-O139 *V. cholerae* are the third most commonly reported group of *Vibrio* spp., and since the year 2000 they accounted for around 40 cases of diarrhoea per year reported to the CDC (*12*). Moreover, in immunocompromised people these bacteria have been associated with a severe form of disease that may lead to sepsis and other life-threatening conditions (*13–15*). The susceptibility pattern of these reported isolates are diverse, showing a varying prevalence of different serovars in different regions.

In Pakistan *V. cholerae* O1 is one of the commonest organisms responsible for diarrhoeal outbreaks (*16*). An outbreak of O139 was also reported in 2002 (*5*). However, data on the isolation rate and antibiotic susceptibility pattern of non-O1 and non-O139 *V. cholerae* are lacking. Such data are valuable for the empirical treatment of patients in special circumstances and to compare with reports from other countries. This study therefore aimed to evaluate the frequency and antimicrobial profile of non-O1 and non-O139 *V. cholerae* in Pakistan from stool samples over the years 1999–2012.

Method

Study design and setting

This was a retrospective analysis of antimicrobial resistance among non-O1 and non-O139 *V. cholerae* strains isolated at the clinical microbiology laboratory of Aga Khan University hospital in Karachi, Pakistan, for diagnostic purposes. This laboratory caters for inpatients as well as outside referrals from other hospitals, clinics and general practitioners across the country. All 95 800 stool samples submitted to the laboratory for analysis from the years 1999 to 2012 were included in the study.

The study protocol and consent procedures were approved by the ethics review committee of the Aga Khan University Hospital. Specific verbal or written consent from patients was not required as the data used were obtained from laboratory records and used anonymously.

Data collection

All stool samples for culture were processed according to standard practice and were inoculated on the following media: MacConkey, *Salmonella-Shigella*, *Campylobacter* and tellurite taurocholate gelatin agar (TTGA) (Oxoid). Stool samples were also inoculated in selenite F broth and alkaline-peptone water and sub-cultured on solid media after overnight incubation. Suspected colonies from TTGA were biochemically confirmed using analytical profile index 20 Enterobacteriaceae (API 20 E) (BioMerieux). *V. cholerae* O1 serogroups were performed using polyvalent O1 antisera, for Ogawa and Inaba strains (Murex Diagnostic) and serogroup O139 (Dienka Sieken). Non-O1 and non-O139 *V. cholerae* were reported when an isolate had a biochemical profile (using API 20 E) of *V. cholerae* but failed to agglutinate by *V. cholerae* antisera O1 and O139.

Antimicrobial drug susceptibility assay was performed by the disk diffusion method using commercially available disks (Becton Dickinson; Sparks Glencoe). The antibiotics tested were: ampicillin (10 µg), tetracycline (30 µg), co-trimoxazole (1.25/23.75 µg), chloramphenicol (30 µg) and ofloxacin (5 µg). Since there were no interpretive breakpoints for *V. cholerae* available in the Clinical and Laboratory Standard Institute guidelines, breakpoints for Enterobacteriaceae were adopted for *V. cholerae* (*17*). *Escherichia coli* (ATCC 25922) strain was used as a quality control.

Data management and statistical analysis

Data extracted from the computerized information system of the hospital were transferred to the statistical software *SPSS*, version 19.0. Frequencies with percentages for age, seasonal distribution and isolation rate were analysed. The resistance pattern of non-O1 and non-O139 *V. cholerae* was evaluated among different age groups.

Results

During the study period (1999–2012), a total of 95 800 stool samples for culture and susceptibility testing were processed at the Aga Khan University

Hospital laboratory. The yield of positive samples (isolation of at least 1 diarrhoea-causing bacterial pathogen including *Salmonella* spp., *Shigella* spp., *V. cholerae*, *Campylobacter* spp. and *Aeromonas* spp.) was 20 125 (21%). Out of these positive cultures 3668 isolates (18%) were *V. cholerae* group (Table 1). Throughout the study period (1999–2012) *V. cholerae* O1 serogroups remained the predominant strain but a changing trend from serotype Ogawa to Inaba was seen in the years 2005–6 and 2012 respectively and again from Inaba to Ogawa was seen in 2007 (Table 1). *V. cholerae* O139 serogroup emerged only in the years 2000–2002.

Of the total *V. cholerae* isolates, 233 (6%) failed to show agglutination by O1 (Ogawa, Inaba) and O139 antisera. These isolates were confirmed as *V. cholerae* by API 20E, and reported as non-O1 and non-O139 *V. cholerae*. Table 1 shows the annual rate of isolation of non-O1 and non-O139 *V. cholerae* over the study period; the highest number of cases were seen in the year 2003.

The majority of stool cultures yielding non-O1 and non-O139 *V. cholerae* (98%) were submitted from different locations of Karachi city. From other locations 1% were isolated from Hyderabad and 0.7% and 0.3% were from Mir Pur Khas and Jacobad respectively. Demographic data showed that 122 cases (52%) of non-O1 and non-O139 *V. cholerae* were in children aged ≤ 14 years and 111 (48%) in those aged > 14 years old, and more cases were in males (135, 58%) than females (98, 42%). Seasonal variations in isolation frequency were observed, with the few or no isolates during winter and a maximum rate during summer with peaks from May to August each year (Figure 1).

The annual trend of antimicrobial susceptibility data revealed increasing resistance to antibiotics such as co-trimoxazole and ampicillin. The lowest rate of resistance was observed against ofloxacin, followed by tetracycline and chloramphenicol, as shown in Figure 2. Table 2 shows that for most antibiotics antimicrobial resistance rates were similar between children and older age groups, except for co-trimoxazole which showed higher resistance among children aged ≤ 14 years than adolescent/adults aged > 14 years (52% versus 33%).

Discussion

To the best of our knowledge this is the first study reporting the frequency of non-O1 and non-O139 *V. cholerae* from clinical samples tested at a diagnostic laboratory of Pakistan. We found that the rate of non-O1 and non-O139 V. cholerae remained at a low level (≤ 10%) of the total *V. cholerae* group throughout the study period except from 2002 to 2004 where an upsurge was observed from

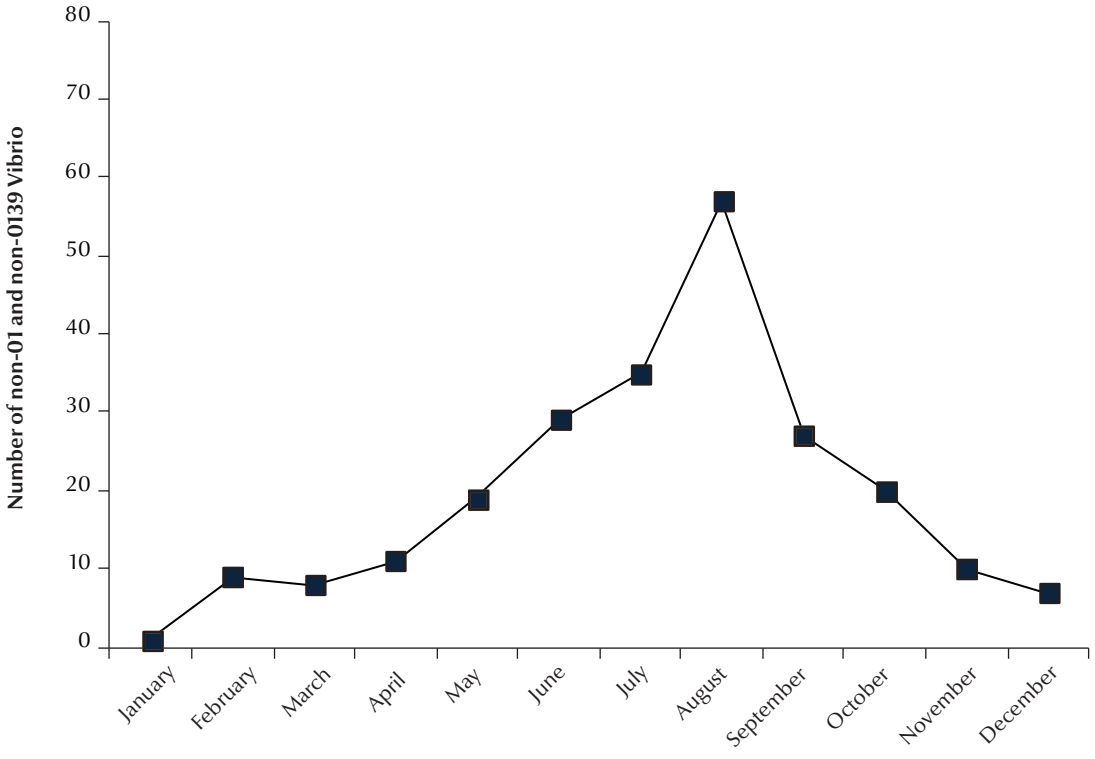

Figure 1 Monthly isolation frequency of non-O1 and non-O139 *Vibrio cholerae* from 1999–2012

Table 1 **Annual isolation rate of non-O1and non-O139 *Vibrio cholerae* at Aga Khan University clinical laboratory; 1999–2012**

Year of culture testing	Stool culture performed	Stool culture growing diarrhoeal pathogens	*V. cholerae* among diarrhoeal pathogens	*V. cholerae* strains				
				O1 (Ogawa)	O1 (Inaba)	O139	Non-O1 and non-O139	
	No.	No.	No.	No.	No.	No.	No.	%
1999	4 807	841	317	282	31	0	4	1
2000	4 806	735	271	213	1	39	18	7
2001	5 587	1 108	412	325	2	81	4	1
2002	8 062	1 334	229	142	3	55	29	13
2003	9 943	2 063	262	182	9	2	69	27
2004	4 700	851	98	64	19	1	14	14
2005	5 556	2 473	738	2	721	0	15	2
2006	6 103	1 441	188	0	181	0	7	4
2007	6 365	1 084	286	149	122	0	15	5
2008	8 267	1 884	238	219	3	0	16	7
2009	9 999	2 667	248	223	5	0	20	8
2010	7 224	1 343	155	143	3	0	9	6
2011	7 597	1 294	152	136	10	0	6	4
2012	6 784	1 007	74	15	52	0	7	10
Total	95 800	20 124	3668	2095	1162	178	233	6

13% to 27%, with a prominent surge in the year 2003.

Our findings of a low isolation rate of non-O1 and non-O139 *V. cholerae* from clinical samples (overall 6% of total *V. cholerae*) is consistent with the sporadic form of diarrhoea caused by endemic strains of non-O1 and non-O139 *V. cholerae* reported from other countries in the region. A literature search showed Indian studies reporting variable isolation frequencies of non-O1 and non-O139 *V. cholerae* from clinical samples. For example, 4% and 5% isolation rates of non-O1 and non-O139 *V. cholerae* were reported from Sevagram in Maharashtra state and Delhi respectively (*18,19*), and higher isolation rates of 13% and 24% from Kolkata and Hubli respectively (*17,20*). Similarly, reports from Thailand showed a lower rate of up to 3% of non-O1 and non-O139 *V. cholerae* among *V. cholerae* isolates (*21*).

The increase in frequency of non-O1 and non-O139 *V. cholerae* noted in 2003 is suggestive of a possible outbreak in that year. Non-O1 and non-O139 *V. cholerae* strains detected during that period also had unique features of co-trimoxazole resistance of 100% compared with a baseline of 35–40% (Figure 2). A plausible association of this high isolation rate could be related to heavy rainfalls leading to nationwide flooding in 2003. These findings are suggestive of a possible emergence of a novel strain with a unique antibiogram, and perhaps increased pathogenicity, leading to symptomatic disease. Due to the retrospective nature of the study we were not been able to perform molecular strain typing and could not retrieve clinical data to confirm the outbreak situation with the novel strain.

Interestingly a majority of non-O1 and non-O139 *V. cholerae* strains were isolated from stool samples received from Karachi, a coastal city which has a questionable supply of clean drinking water and high rates of enteric fever and other gastrointestinal infections in the community (*22,23*). Additionally a lack of proper surveillance of diarrhoeal outbreaks leads to paucity of data. Similar upsurges

Table 2 **Frequency of non-O1 and non-O139 *Vibrio cholerae* isolation and antimicrobial resistance rates, by age group**

Age (years)	Isolation frequency	Antimicrobial resistance									
		Co-trimoxazole		Ampicillin		Tetracycline		Chloramphenicol		Ofloxacin	
	No.	No.	%	No	%	No	%	No	%	No	%
≤ 14	122	63	52	12	10	1	1	4	3	0	0
> 14	111	37	33	13	12	4	4	0	0	1	1

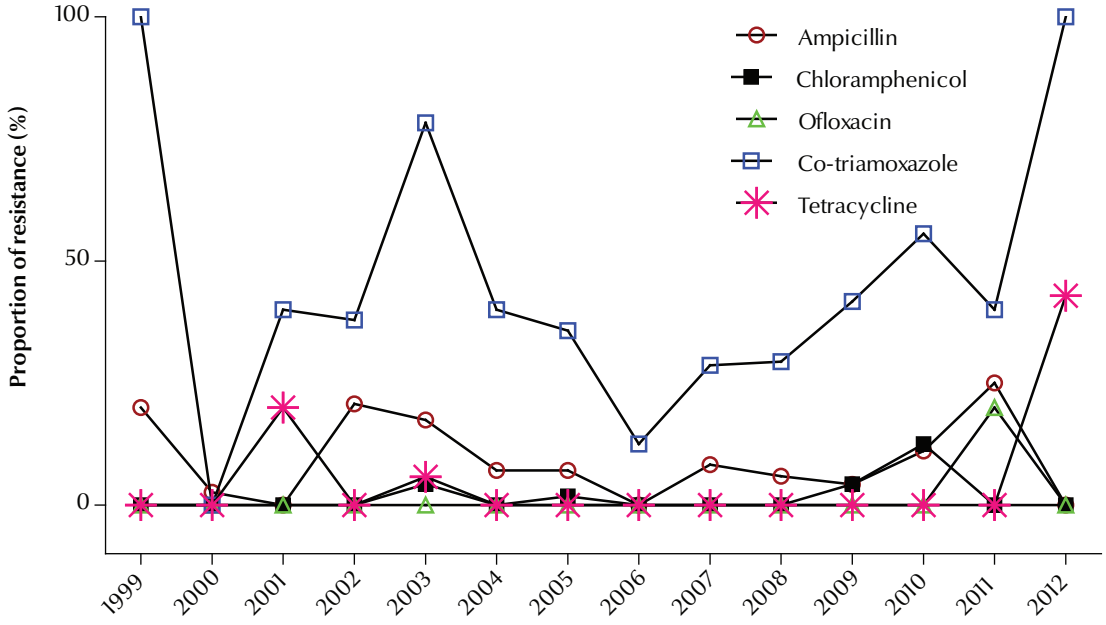

Figure 2 Annual antimicrobial resistance pattern of non-O1 and non-O139 *Vibrio cholerae* from 1999–2012

in the isolation rate of non-O1 and non-O139 *V. cholerae* have been reported from India during the period 2002–04, showing replacement of predominant *V. cholerae* O1 serotypes (*20*). These findings suggest the possibility of a regional outbreak with novel serotypes and emphasizes the need for continuous surveillance and monitoring of regional diarrhoeal strains.

Throughout the duration of the current study (1999–2012) *V. cholerae* O1 serogroups remained the predominant serotype. Another interesting finding of our study is related to *V. cholerae* O139. It seems that a new serotype with a different antibiogram (*5*) had emerged in the year 2000–02. This is comparable with the similar trend reported from India (*19*). Further comparison of previously published antimicrobial susceptibility data of *V. cholerae* O139 from Pakistan (*5*) is comparable with India (*19*) and strains from both regions had a similar susceptibility pattern to reported antimicrobials including co-trimoxazole (*5,19*). This again highlights the likelihood of circulation of particular type of *V. cholerae* O139 in the Indian subcontinent during that period. Non-O1 and non-O139 *V. cholerae* were likely to be the origin of the new virulence gene, as emergence of the O139 serovar resulted due to horizontal gene transfer between *V. cholerae* O1 and O22 (*24,25*). Therefore, continuous monitoring of these serovars will provide valuable information on mechanisms of virulence.

Finally, the current data showed a fluctuating pattern of susceptibility from year to year of non-O1 and non-O139 *V. cholerae* isolates to the antibiotics commonly used against cholera. Low resistance to tetracycline was seen except in the year 2012. Resistance to co-trimoxazole peaked in 2003 and 2012. Therefore with empirical use of World Health Organization recommended drugs such as tetracycline and doxycycline intended for treatment of cholera, continuous monitoring of antimicrobial resistance surveillance should be maintained.

Conclusion

In conclusion, this study found that, despite annual fluctuations, the rate of non-O1 and non-O139 *V. cholerae* remained a significant cause of diarrhoeal illness in Pakistan, as it is in neighbouring regions, with the possibility of emergence of novel pathogenic strains. These findings emphasize the need for continuous surveillance of serotypes and monitoring of antimicrobial resistance of regional diarrhoeal strains to predict future epidemics and define measures to curtail the disease. Surveillance data will also provide baseline information to develop empirical antibiotic choice, effective vaccines and preventive strategies.

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