Determinants of Virulence and Antimicrobial Susceptibility in Non-O1, Non-O139 *Vibrio cholerae* Isolates

Anabel Fernández-Abreu MS, Laura Bravo-Fariñas PhD, Giselle Rivero-Navea MD, Nereyda Cabrera-Cantelar MD MS, Fidel A. Nuñez-Fernández MD PhD, Yanaika Cruz-Infante, Adalberto Águila-Sánchez MD, Jenny L. Hernández-Martínez

ABSTRACT

INTRODUCTION In the Caribbean region, acute diarrheal diseases caused by the *Vibrio* genus have increased in recent years, following the 2010 earthquake in Haiti. Based on its capsular lipopolysaccharide, *Vibrio cholerae* is classified into more than 200 serogroups, divided into O1, O139 and non-O1, non-O139. Non-O1 serogroups produce clinical features ranging from mild diarrhea to severe dehydration. In Cuba, circulation of non-O1, non-O139 *V. cholerae* has been reported both in outbreaks and sporadic cases.

OBJECTIVE Describe the antimicrobial susceptibility of *V. cholerae* to the drugs of interest used in its treatment and verify the presence of enzymatic virulence factors.

METHODS A descriptive, cross-sectional study was conducted in January through November 2014, based on 125 non-O1, non-O139 *V. cholerae* isolates obtained during 2013 and 2014 from patients with acute diarrheal disease (isolates from the National Reference Laboratory for Acute Diarrheal Diseases of the Pedro Kourí Tropical Medicine Institute, Havana). Bacteriological identification was performed according to conventional methods. Antimicrobial susceptibility was determined by the Bauer-Kirby agar diffusion method. The technique described by Robinson in 1986 was used to determine virulence factors, hemolytic activity and enzyme factors (DNase, elastase, gelatinase), and Karagozova's method for the enzyme lecithinase.

RESULTS Highest percentages of sensitivity were obtained for azithromycin (98.4%), doxycycline (97.6%) and ciprofloxacin (96.8%), and highest resistance values for ampicillin (60%), sulfonamide (46.4%) and trimethoprim–sulfamethoxazole (32%). Six resistance patterns were detected, four found in Cuba for the first time, as well as six patterns of multidrug resistance (4.8%). All isolates had at least two extracellular enzymes as virulence factors. The most frequent were gelatinase (90.4%) and lecithinase (77.6%). The percentage of virulence factors was lower in the group of isolates resistant to ≥2 antimicrobials. An inverse relationship was found between presence of enzymatic virulence factors and resistance in the isolates studied.

CONCLUSIONS The results suggest that azithromycin, doxycycline and ciprofloxacin should continue to be used for treatment of *V. cholerae*-caused infections, and confirmed the presence of four new resistance patterns in isolates circulating in Cuba.

KEYWORDS *Vibrio cholerae*, cholera, antimicrobial resistance, multiple drug resistance, multidrug resistance, virulence, virulence factors, pathogenicity, Cuba

INTRODUCTION

In the Caribbean region, acute diarrheal diseases (ADD) caused by the *Vibrio* genus have increased in recent years, following the earthquake in Haiti in 2010, after which WHO recommended strengthening epidemiological surveillance and national preparedness in neighboring countries to detect outbreaks immediately.[1] In June 2012, the Cuban Ministry of Public Health (MINSAP) reported a cholera outbreak in Granma Province, with 417 confirmed cases and 3 deaths. In August 2013, a new outbreak occurred in the provinces of Havana, Santiago and Camagüey, with more than 163 cases reported to PAHO.[2] In 2015, 386,507 ADD cases were reported, of which 65 were confirmed to be cholera, while in 2016, 288,832 cases of ADD and no cholera cases were reported.[3,4]

Vibrio cholerae is classified by its capsular lipopolysaccharide in more than 200 serogroups, divided into O1, O139 and non-O1, non-O139, of which only O1 and O139 are epidemic. *V. cholerae* O1 is biochemically identical to non-O1, non-O139 *V. cholerae*.[5,6] The latter are isolated in patients with clinical manifestations ranging from mild diarrhea to severe dehydration, but do not present epidemic potential. In addition, they are associated with extraintestinal symptoms.[5,7] In Cuba, the circulation of non-O1, non-O139 *V. cholerae* has been reported in outbreaks and sporadic cases.[8,9]

The pathogenic mechanisms involved in diarrhea caused by these enteropathogens are still not well established; a different pathogenesis is proposed for nonepidemic serogroups, in which heterogeneous virulence factors intervene.[8,10,11] In Latin America and the Caribbean, there are few studies on virulence factors in isolates of non-O1, non-O139 *V. cholerae* from patients with ADD.[8] Extracellular products are described, such as a thermostable enterotoxin NAG-ST, pili coregulating toxin and the production of extracellular enzymes such as gelatinase, elastase and lecithinase.[11] Diarrhea caused by *V. cholerae* can be treated with hydration, and antimicrobials, if necessary. To date there are no vaccines for these serogroups.[12]

The emergence of antimicrobial resistance is a global phenomenon. International studies have described *V. cholerae* isolates resistant to multiple antimicrobials, with increased resistance to furazolidone, nalidixic acid, sulfisoxazole, streptomycin and trimethoprim–sulfamethoxazole, and decreased sensitivity to ciprofloxacin.[1,12] Multidrug resistance (MDR) is common in strains of *V. cholerae* isolated from patients with intestinal and extraintestinal infections, so carrying out antimicrobial susceptibility studies is necessary and useful for establishing successful therapies.[13,14]

Determination of *V. cholerae* antimicrobial susceptibility is not serogroup specific. Cutoff points established by the Clinical Laboratory Standards Institute (CLSI) are determined for the species, regardless of serogroups, since intrinsic resistance to antimicrobials is associated with acquisition of genetic determinants of resistance and mutations, within species and among enteropathogens in general.[14,15]

Some antimicrobial susceptibility studies of *V. cholerae* have been done in Cuba. In 2004, non-O1, non-O139 *V. cholerae* isolates from several Cuban provinces were obtained, showing resistance to ampicillin (14%) and trimethoprim–sulfamethoxazole (16%), while 98% showed sensitivity to tetracycline and 96% to chloramphenicol.[16] A 2008 study found 32.3% and 30.7% of isolates resistant to sulfonamide and ampicillin, respectively, and percentages of sensitivity against the antibiotics doxycycline, chloramphenicol, tetracycline, trimethoprim–sulfamethoxazole, ciprofloxacin and nalidixic acid exceeded 85%.[17] In 2016, 144 isolates of *V. cholerae* O1 El Tor biotype, Ogawa serotype, from outbreaks in the provinces of Granma and Havana in June 2012 through January 2013 were studied. Isolates showed resistance values above 90% for trimethoprim–sulfamethoxazole, sulfonamide and ampicillin. Intermediate sensitivity was found to ciprofloxacin and chloramphenicol (30.6% and 27.1%, respectively). Sensitivity levels higher than 92% were observed for azithromycin, doxycycline, gentamicin and tetracycline. No multidrug-resistant strains were identified.[18]

Non-O1, non-O139 V. cholera is considered an international emerging pathogen. Although phenotypic characterization studies of these serogroups have been conducted since the 1980s, the relationship between virulence determinants and antimicrobial resistance in non-O1, non-O139 V. cholerae in Cuba is unknown. Because ADD caused by Vibrio species constitutes an important challenge for the Cuba's health system, we set out to describe antimicrobial susceptibility to drugs commonly used in ADD treatment and verify the presence of enzymatic virulence factors.

METHODS

Design and population A cross-sectional descriptive study was conducted in January through November 2014, based on 125 non-O1, non-O139 *V. cholerae* isolates obtained from feces of patients with ADD in Cuba in 2013 and 2014. The isolates, kept in Pasteur conservation medium, belonged to the microbial culture collection of the National Reference Laboratory for Acute Diarrheal Diseases at IPK.

Variables *Antimicrobial susceptibility* was classified as sensitive, intermediate and resistant. Isolates were classified as sensitive if they were inhibited by antimicrobial concentrations reached at the recommended dose for the infection site. They were considered of intermediate sensitivity when they displayed minimum inhibitory concentrations close to the antimicrobial levels reached in blood or tissues with a lower degree of response than the sensitive strains. The intermediate category implies clinical efficacy in sites of the body where antimicrobials are physiologically concentrated or when the drug can be used in higher than normal doses. It also includes a buffer zone that could help avoid small technical factors (subjectivity in reading inhibition halos) that are difficult to control and may cause important discrepancies in interpretation, especially for drugs with narrow pharmacotoxic margins. Isolates were classified as resistant if they were not inhibited by serum antimicrobial concentrations achieved at standard doses or had minimal inhibitory concentrations in the range where specific resistance mechanisms are present and clinical efficacy is not demonstrable.

A *resistance pattern* was defined by resistance to one or more antimicrobial, of any family. *MDR* was defined by resistance to three or more antimicrobial families, as established by CLSI, 2017.[15] *Virulence factors* studied were beta-hemolysin, DNase, elastase, gelatinase and lecithinase.[11]

Antimicrobial families analyzed were beta-lactams (ampicillin), tetracyclines (doxycycline, tetracycline), sulfonamides (sulfonamide, trimethoprim–sulfamethoxazole), phenicols (chloramphenicol), macrolides (azithromycin), aminoglycosides (amikacin) and quinolones (ciprofloxacin).

Procedures Bacteriological identification was performed by conventional methods. Biochemical tests for the use of amino acids and carbohydrates for genus confirmation were done by the Moeller method. For species identification, isolates were tested for sodium chloride tolerance and sucrose uptake. Serological tests were performed by slide agglutination with O1 and O139 *V. cholerae* polyvalent antisera.[19]

The Bauer–Kirby agar diffusion method was used,[20] following CLSI 2010 criteria,[21] to test for isolates' susceptibility to antimicrobials of choice and alternatives: ampicillin, doxycycline, tetracycline, sulfonamide, trimethoprim–sulfamethoxazole and chloramphenicol. Criteria established for the *Enterobacteriaceae* family were used for azithromycin and alternative antimicrobials such as amikacin and ciprofloxacin.[15]

Robinson's technique was used to detect virulence factors beta-hemolysin, DNase, elastase and gelatinase,[22] Karagozova's for enzyme lecithinase.[23]

Analysis Data were entered and processed using Excel. Absolute and relative frequencies were calculated for each of the study variables.

Ethics Data management procedures ensured confidentiality of patients whose isolates were tested. The study was approved by the IPK Ethics Committee.

RESULTS

Antimicrobial susceptibility Only 2 (1.6%) of the 125 isolates, showed sensitivity to all antimicrobials studied. Sensitivities of >96% were found to ciprofloxacin, doxycycline and azithromycin. Resistance was most frequent to ampicillin (60%), sulfonamides (46.4%) and trimethoprim–sulfamethoxazole (32%) (Table 1).

Resistance patterns Table 2 displays isolates' distribution by resistance pattern. Six resistance patterns were found, four of them detected for the first time in Cuba (ampicillin + sulfonamide, ampicillin + tetracycline, ampicillin + ciprofloxacin, and sulfonamides + trimethoprim–sulfamethoxazole), as well as six MDR patterns (4.8%).

Virulence factors The proportions of virulence-factor positive isolates were: hemolysins 61.6% (77/125 isolates), DNase 60% (75), elastase 48.8% (61), gelatinase 90.4% (113) and lecithinase 77.6% (97).

Enzymatic virulence factors and resistance All monoresistant isolates expressed virulence factors, but proportions were smaller in isolates resistant to ≥2 microbials: hemolysins 66.3% (65/98), DNase 61.2% (60), elastase 46.9% (46), gelatinase 70.4% (69) and lecithinase 77.6% (76).

Table 1: Antimicrobial susceptibility of non-O1, non-O139
***V. cholerae* isolates (n = 125)**

Antimicrobial	Resistant n (%)	Intermediate n (%)	Sensitive n (%)
Ampicillin	75 (60.0)	13 (10.4)	37 (29.6)
Ciprofloxacin	3 (2.4)	1 (0.8)	121 (96.8)
Chloramphenicol	4 (3.2)	6 (4.8)	115 (92.0)
Doxycycline	1 (0.8)	2 (1.6)	122 (97.6)
Tetracycline	5 (4.0)	6 (4.8)	114 (91.2)
Trimethoprim–sulfamethoxazole	40 (32.0)	0 (0.0)	85 (68.0)
Sulfonamide	58 (46.4)	3 (2.4)	64 (51.2)
Amikacin	10 (8.0)	1 (0.8)	114 (91.2)
Azithromycin	0 (0.0)	2 (1.6)	123 (98.4)

Table 2: Patterns of resistance and MDR of non-O1, non-O139
***V. cholerae* isolates (n = 125)**

Resistance[a]	n (%)
AMP, SXT, SUL[b]	34 (27.2)
AMP, SXT	26 (20.8)
AMP, SUL[c]	17 (13.6)
SXT, SUL[c]	9 (7.2)
AMP, TET[c]	4 (3.2)
AMP, CIP[c]	2 (1.6)
Multidrug resistance[d]	
AMP, CAM, TET	1 (0.8)
AMP, SUL, CAM	1 (0.8)
AMP, SXT, CAM	1 (0.8)
AMP, SXT, SUL, AK	1 (0.8)
AMP, CIP, SXT, SUL	1 (0.8)
AMP, SXT, CAM, TET, SUL, DOX	1 (0.8)

[a]resistance to ≥1 antimicrobials in any family
[b]SXT and SUL belong to same family
[c]new pattern   [d]resistance to ≥3 antimicrobial families
AMK: amikacin   AMP: ampicillin   CAM: chloramphenicol   CIP: ciprofloxacin
DOX: doxycycline   MDR: multidrug resistance   SUL: sulfonamide
SXT: trimethoprim–sulfamethoxazole   TET: tetracycline

DISCUSSION

Although this study found sensitivities exceeding 95% for the most widely used antibiotics for treatment of *V. cholerae* diarrhea (doxycycline, azithromycin and ciprofloxacin), there is recent evidence of increased antimicrobial resistance among *V. cholerae* strains to commonly used drugs, implying serious treatment challenges worldwide.[24]

Our observed high rates of sensitivity to azithromycin echo previous findings reported in *V. cholerae* isolates from hospitals in Dhaka and Matlab, Bangladesh in 2000–2012, when 95% of isolates were azithromycin sensitive.[25] Mahmud's study of isolates from a 2012 outbreak in Sierra Leone found 100% sensitivity to the same antimicrobial.[26] A publication based on testing 144 Cuban isolates of *V. cholerae* O1 from outbreaks reported 99.3% (143/144) sensitivity to azithromycin.[18] It is recommended as first-line therapy only for pediatric patients and pregnant women with suspected cholera, and as second-line in other cases.[27]

We found sensitivities to doxycycline similar to those described in Cuba by Fernández in 2016.[18] However, continued monitoring of its effectiveness in treating *V. cholerae* is important, since it

is the first-line cholera treatment in Cuba and is also used for contact chemoprophylaxis.

Ciprofloxacin sensitivity was similar to that published by Mandal in India and Murhekar in New Guinea, 96.8% and 99%, respectively. [28,29] Sensitivities of 100% have been obtained in Cuba, by Bravo in 2006[8] and Cabrera in 2008.[17] These high sensitivities may be due to absence of mutations in the gyrA and parC genes or Cuba's policy on treatment of ADD caused by *V. cholerae*, since ciprofloxacin is indicated in adult patients with compromised oral route or a history of allergy to the drug of choice (per MINSAP's Cholera Control Program).[30]

The chloramphenicol sensitivities we observed were similar to those seen by Murhekar in New Guinea.[29] In Cuba, chloramphenicol is reserved for treatment of serious life-threatening infections, when there are no effective and less toxic treatment alternatives. [27] An interesting finding not previously reported in Cuba was identification of 4 (3.2%) isolates resistant to chloramphenicol. Thapa found 9.1% chloramphenicol resistance in Nepal in 2015.[6]

There was a high frequency of sensitivity to tetracycline, although somewhat less than the 100% published by Bakhshi in Iran in 2014.[31] In Cuba, Bravo found 98% of non-O1, non-O139 *V. cholerae* isolates were sensitive to tetracycline in a 10-year study (1996–2005).[8]

Aminoglycosides are a class of commonly used antimicrobials effective in clinical practice. Although there are several mechanisms, they are still active against the bulk of gram-negative aerobic bacilli, and amikacin can be used in cases of resistance. [27] The >90% sensitivity we found for amikacin is comparable to the 88.2% Rashed observed in Dhaka.[32] This is in sharp contrast to much lower sensitivity (8.3%) to amikacin in non-O, non-O139 *V. cholerae* reported by Bakhshi in Iran.[31]

Among the antimicrobials we studied, the highest percentage of resistance was observed for ampicillin. Bravo in Cuba and Dutta in India found 55.5% of *V. cholerae* isolates resistant to ampicillin,[9,33] slightly lower than the 60% observed in our study. In contrast, Thapa found 100% of O1 *V. cholerae* isolates resistant to this antimicrobial,[6] as did Shrestha in India and Talkington in *V. cholerae* isolates from Haiti.[34,35] These results could be due to production of extended-spectrum beta-lactamase; Gosh found this enzyme in 92.9% (52/56) of ampicillin-resistant *V. cholerae* isolates. [12] There is overwhelming evidence that emergence of antibiotic resistance is conditioned by selective pressure on the microbial flora because of inappropriate or excessive use of antibiotics.[36]

Four of the six resistance patterns we found were identified for the first time in Cuba. In 2006, Bravo's Cuban study found some resistance patterns similar to those we observed (e.g., ampicillin + sulfonamide + trimethoprim–sulfamethoxazole),[8] and Bueno's 2011 study (in Cuba) of 63 isolates of non-O1, non-O139 *V. cholerae* found that the predominant pattern was chloramphenicol + trimethoprim–sulfamethoxazole.[37] While the frequency of MDR isolates was low (<5%), in 2016, Fernández found no MDR.[18] Our finding of MDR could be due to the presence of integrons, natural gene acquisition systems that help bacteria capture exogenous genes and incorporate them into their genome, playing a prominent role in spread of resistance, because they often carry genes associated with mobile genetic

elements.[12] Bakhshi suggests there may be class 1 integrons in enteropathogens.[31]

The presence of virulence factors may be due to their production by microorganisms of cellular proteins to invade the host immune system and achieve its colonization, since *V. cholerae* has several host colonization and infection mechanisms.[11] In addition to the cholera toxin, numerous extra and intracellular proteins are responsible for virulence, pathogenicity and cytotoxicity of *V. cholerae* O1, O139 and non-O1, non-O139.[17] In 2006, Cabrera (Cuba) found hemolysin and gelatinase in all isolates, DNase in 73.8%, lecithinase in 80%, and elastase in 86.1%.[17] Also in Cuba, Bueno found higher percentages than ours for gelatinase (96.8%) and hemolysin (92.1%), and lower for elastase (79.4%), lecithinase (73%) and DNase (68.3%).[37]

Each virulence factor was more frequent in monoresistant isolates than in those resistant to more than one antimicrobial. Other studies have related virulence to the presence of various virulence factors: Bina (USA) reported that *V. cholerae* isolates showing resistance–nodulation–division efflux pumps displayed decreased production of cholera toxin and pili coregulating toxin compared to wild-type strains, explained by reduced transcription of tcpP and toxT.[38] However, Spengler held that the presence of VexH efflux pumps in *V. cholerae* contributed to production of cholera toxin and pili coregulating toxin.[39]

It has been suggested that porin deficiency in *Klebsiella pneumoniae* increases antimicrobial resistance and decreases virulence. Similar results are reported for *Neisseria meningitidis*, *Pseudomona aeruginosa*, *Vibrio* spp and other *Enterobacteriaceae*. Studies suggest an inverse association between resistance and virulence, but the cause of this association is still unknown. Some authors suggest that it may be because less pathogenic serotypes maintain colonization for a longer time and are more exposed to multiple antimicrobials.[38,40,41]

One of the study's limitations was lack of testing for minimal inhibitory concentration in resistant isolates. Nor was it possible to determine mechanisms of resistance in the isolates studied. Nevertheless, the results contribute to knowledge about the emergence and dissemination in Cuba of isolates resistant to various antimicrobials, and further our understanding of the determinants of virulence. It is advisable to maintain microbiological surveillance of non-O1, non-O139 *V. cholerae* in Cuba and to develop new studies to identify other virulence factors in these serogroups.

CONCLUSIONS

The results support continued use of the antimicrobials azithromycin, doxycycline and ciprofloxacin for treatment of infections caused by *V. cholerae,* and confirm the existence of four new resistance patterns in isolates circulating in Cuba. -⋀-

REFERENCES

1. World Health Organization [Internet]. Geneva: World Health Organization; c2014. Mediacenter. Fact sheets. Cólera; 2012 [cited 2015 Dec 10]. Available from: http://www.who.int/mediacentre/factsheets/fs107/es/
2. Zelada Valdés A, Ledón Pérez TY, Fando Calzada RA. El cólera: una enfermedad infecciosa reemergente. El candidato vacunal cv 638, una herramienta para su prevención. Rev CENIC. 2015 May–Aug;46(2):131–43. Spanish.
3. National Health Statistics and Medical Records Division (CU). Anuario Estadístico de Salud 2016 [Internet]. Havana: Ministry of Public Health (CU); 2017 [cited 2017 May 20]. Available from: http://files.sld.cu/dne/files/2017/05/Anuario_Estad%C3%ADstico_de_Salud_e_2016_edici%C3%B3n_2017.pdf. Spanish.
4. Pan American Health Organization. Actualización Epidemiológica: Cólera 23 de nov 2016. Washington D.C.: Pan American Health Organization; World Health Organization; 2016. Spanish.
5. González Fraga S, Villagra de Trejo A, Pichel M, Figueroa S, Merletti G, Caffer MI, et al. Caracterización de aislamientos de V. cholerae no-O1, no-O139 asociados a cuadros de diarrea. Rev Argent Microbiol. 2009 Jan–Mar;41(1):11–9. Spanish.
6. Thapa Sherestha U, Adhikari N, Maharjan R, Banjara MR, Rijal KR, Basnyat SR, et al. Multidrug resistant Vibrio cholerae O1 from clinical and environmental samples in Kathmandu city. BMC Infect Dis. 2015 Feb 27;15:104.
7. Farmer JJ III, Michael J, Brenner FW, Cameron DN, Birkhead KM. Taxonomic outline of the prokaryotes. In: Bergey's Manual of Systematic Bacteriology. 2nd ed. Release 4.00 [Internet]. 2003 Oct [cited 2014 Nov 11]. Available from: https://www.bergeys.org/outlines/bergeysoutline_4_2003.pdf
8. Bravo Fariñas L, Fernández A, Ramírez MM, Llop A, Martínez G, Hernández RI, et al. Caracterización microbiológica de cepas de V. cholerae no-O1 aisladas en Cuba. Rev Cubana Med Trop [Internet]. 2007 Sep–Dec [cited 2014 Jan 25];59(3):227–33. Available from: http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S0375-07602007000300008. Spanish.
9. Bravo L, Majano A, Fernández A, Ramírez MM, Águila A, Cruz Y. Susceptibilidad antimicrobiana de bacilos gramnegativos anaerobios facultativos oxidasa positiva. Arch Venezolanos Farmacol Terapéutica. 2009;26(1):23–7. Spanish.
10. Rojas N, Chaves E, García F. Bacteriología Diagnóstica. Costa Rica. Non-O1 and Non-O139 Vibrio cholerae Infections [Internet]. San José de Costa Rica: University of Costa Rica; 2006 [cited 2014 Jan 25]. 150 p. Available from: http://www.infectologiapediatrica.com/attachments/bacteriologia_diagnostica.pdf. Spanish.
11. Baq PK, Bhowmik P, Hajra TK, Ramamurthy T, Sarkar P, Majumder M, et al. Putative virulence traits and pathogenicity of V.cholerae Non-O1, Non-O139 isolates from surface waters in Kolkata, India. Appl Environ Microbiol. 2008 Sep;74(18):5635–44.
12. Ghosh A, Ramamurthy T. Antimicrobials & cholera: are we stranded? Indian J Med Res. 2011 Feb;133(2):225–31.
13. Ceccarelli D, Alam M, Huq A, Colwell RR. Reduced susceptibility to Extended-Spectrum β-Lactams in V. cholerae isolated in Bangladesh. Front Public Health. 2016 Oct 18;4:231.
14. Yu L, Zhou Y, Wang R, Lou J, Zhang L, Li J, et al. Multiple antibiotic resistance of V. cholerae serogroup O139 in China from 1993 to 2009. PLoS One. 2012:7(6):e38633.
15. Clinical and Laboratory Standards Institute (CLSI). M100 Performance Standards for Antimicrobial Susceptibility Testing. 27th ed. Pennsylvania: Clinical and Laboratory Standards Institute; 2017 Jan 1. 224 p.
16. Bravo L, Cabrera R, Cabrera LE, Ramírez M, Castañeda N, Fernández A, et al. Sensibilidad antimicrobiana en cepas de Vibrio cholerae no-O1 aisladas en Cuba. Rev Esp Quimioterap. 2004;17(2):200–1. Spanish.
17. Cabrera Rodríguez LE, Bravo Fariñas L, Ramírez Álvarez M, Llop Hernández A, Fernández Abreu A, Morier Díaz L, et al. Susceptibilidad a los antimicrobianos y factores de virulencia en cepas de V. cholerae no-O1 aisladas de pacientes con enfermedad diarreica aguda. Rev Biomed. 2008 Sep–Dec;19(3):138–44. Spanish.
18. Fernández Abreu A, Bravo Fariñas L, Águila Sánchez A, Cruz Infante Y, Illnait Zaragozí MT, Llop Hernández A, et al. Susceptibilidad antimicrobiana en aislamientos cubanos de Vibrio cholerae O1 procedentes de muestras clínicas. Rev Cubana Med Trop. 2016 Apr;68(1):51–8. Spanish.
19. Win W Jr, Allen S, Janda W, Koneman E, Procop G, Schreckenberg P, et al. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2008.
20. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Path. 1966 Apr;45(4):493 –6.
21. Clinical and Laboratory Standard Institute (CLSI). Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; Approved Guideline–Second Edition. Pennsylvania: Clinical and Laboratory Standard Institute (CLSI); 2010 Aug. 12 p.
22. Robinson J, Beaman J, Wagener L, Burke V. Comparison of direct plating with the use of enrichment culture of isolating of Aeromonas spp from faeces. J Med Microbiol. 1986 Dec;22(4):315–7.
23. Karagozova AV, Salnikova OI. [The expression of the pathogenic properties of the V. cholerae O139 serogroup in vitro]. Zh Mikrobiol Epidemiol Immunobiol. 2000 May–Jun;(3):7–10. Russian.
24. Gupta PK, Pant ND, Bhandari R, Shrestha P. Cholera outbreak caused by drug resistant Vibrio cholerae serogroup O1 biotype El Tor serotype Ogawa in Nepal; a cross-sectional study. Antimicrob Resist Infect Control. 2016 Jun 4;5:23
25. Kumar Das S, Klontz EH, Azmi IJ, Ud-Din AIMS, Chisti MJ, Afrad MH, et al. Characteristics of Mul-

tidrug Resistant Shigella and Vibrio cholerae O1 Infections in Patients Treated at an Urban and a Rural Hospital in Bangladesh ISRN Microbiology. Int Scholarly Res Notices [Internet]. 2013 [cited 2013 Nov 10]; 2013(Article ID 213915). 8 p. Available from: https://www.hindawi.com/journals/isrn/2013/213915/

26. Mahmud ZH, Islam S, Zaman RU, Akter M, Talukder KA, Bardhan PK, et al. Phenotypic and genotypic characteristics of Vibrio cholerae O1 isolated from the Sierra Leone cholera outbreak in 2012. Trans R Soc Trop Med Hyg. 2014 Nov;108(11):715–20.

27. Calvo-Barbado DM, Delgado-Martínez I. Formulario Nacional de Medicamentos. [Internet]. Havana: Editorial Ciencias Médicas; 2014 [cited 2014 Jan 25]. Available from: http://www.bvs.sld.cu/libros_texto/formulario_nac_medicamentos_4taed/indice_p.htm. Spanish.

28. Mandal J, Dinoop KP, Parija SC. Increasing antimicrobial resistance of V. cholerae OI biotype EI Tor strains isolated in a tertiary-care centre in India. J Health Popul Nutr. 2012 Mar;30(1):12–6.

29. Murhekar M, Dutta S, Ropa B, Dagina R, Posanai E, Rosewell A. V. cholerae antimicrobial drug resistance, Papua New Guinea, 2009–2011. Western Pac Surveill Response J. 2013 Sep 2;4(3):60–2.

30. Ministry of Public Health (CU). Programa Nacional para el Control del Cólera. Havana: Ministry of Public Health (CU); 2010. Spanish.

31. Bakhshi B, Eftekhari N, Pourshafie MR. Genetic elements associated with antimicrobial resistance among intestinal bacteria. Jundishapur J Microbiol. 2014 May;7(5):e9924.

32. Rashed SM, Mannan SB, Johura FT, Islam MT, Sadique A, Watanabe H, et al. Genetic characteristics of drug-resistant V. cholerae O1 causing endemic cholera in Dhaka, 2006 –2011. J Med Microbiol. 2012 Dec;61(Pt 12):1736–45.

33. Dutta D, Chowdhury G, Pazhaniv G, Guin S, Dutta S, Ghosh S, et al. V. cholerae Non-O1, Non-O139 serogroups and cholera-like diarrhea, Kolkata, India. Emerg Infect Dis [Internet]. 2013 [cited 2014 Jan 25]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3647666/

34. Shrestha SD, Malla S, Adhikari BR, Shakya G, Basnyat SR, Sharma S. Antibiotic susceptibility patterns of V. cholerae isolates. J Nepal Med Assoc. 2010 Jul–Sep;49(179):232–6.

35. Talkington D, Bopp C, Tarr C, Parsons MB, Dahourou G, Freeman M, et al. Characterization of Toxigenic V. cholerae from Haiti, 2010–2011. Emerg Infect Dis. 2011 Nov;17(11):2122–9.

36. Guanche Garcell H, Pisonero Socias JJ, Enseñat Sánchez R, Fiterre Lancis I, Mir Narbona I, García Arzola B, et al. Impacto de un programa de control de la calidad de la prescripción de antibióticos en un hospital de La Habana. Rev Panam Salud Pública. 2011 Dec;30(6):598–602. Spanish.

37. Bueno Y. Caracterización fenotípica de cepas de V. cholerae no-O1 aisladas de pacientes con enfermedad diarreica aguda [thesis]. [Havana]: Dr Pedro Kourí Institute of Tropical Medicine (IPK); 2011. Spanish.

38. Bina XR, Provenzano D, Nguyen N, Bina JE. Vibrio cholerae RND family efflux systems are required for antimicrobial resistance, optimal virulence fact or production, and colonization of the infant mouse small intestine. Infect Immun. 2008 Aug;76(8):3595–605.

39. Spengler G, Kincses A, Gajdács M, Amaral L. New Roads Leading to Old Destinations: Efflux Pumps as Targets to Reverse Multidrug Resistance in Bacteria. Molecules. 2017 Mar 15;22(3):468. pii: E468.

40. Beceiro A, Tomás M, Bou G. Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? Clin Microbiol Rev. 2013 Apr;26(2):185–230.

41. Díaz Rigau L, Cabrera Rodríguez LE, Fernández Núñez T, Arias Vegas M, Scull Scull G. Serogrupos y susceptibilidad antimicrobiana en cepas de Shigella. Rev Cubana Pediatr. 2009 Jan–Mar;81(1):1–9. Spanish.

THE AUTHORS

Anabel Fernández-Abreu (Corresponding author: anabel@ipk.sld.cu), microbiologist with a master's degree in bacteriology and mycology. Adjunct researcher, National Reference Laboratory for Acute Diarrheal Diseases, Pedro Kourí Tropical Medicine Institute (IPK), Havana, Cuba.

Laura Bravo-Fariñas, biologist with a doctorate in health sciences. Consulting professor and senior researcher, IPK, Havana, Cuba.

Giselle Rivero-Navea, physician specializing in microbiology, IPK, Havana, Cuba.

Nereyda Cabrera-Cantelar, physician specializing in microbiology, with a master's degree in bacteriology and mycology, IPK, Havana, Cuba.

Fidel A. Nuñez-Fernández, physician specializing in parasitology with a doctorate in medical sciences, IPK, Havana, Cuba.

Yanaika Cruz-Infante, health technologist, IPK, Havana, Cuba.

Adalberto Águila-Sánchez, physician specializing in microbiology. Adjunct researcher, IPK, Havana, Cuba.

Jenny L. Hernández-Martínez, chemistry technician, IPK, Havana, Cuba.