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Antibiotic resistance patterns amongst clinical *Vibrio cholerae* O1 isolates from Accra, Ghana

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Abstract

One of the protocols in the treatment and control of cholera infection is antibiotic therapy. However, increasing rates of antibiotic resistance amongst enteric bacteria including *Vibrio cholerae* have been reported in recent times. There has been no continuous surveillance of antibiotic susceptibility profiles for *V. cholerae* O1 in Ghana. This study determined resistance profiles of *V. cholerae* O1 to selected and commonly used antimicrobial agents and assessed resistance patterns across year periods. Additionally, the range of antibiotics currently effective for treatment and infection control during cholera outbreaks was ascertained. We screened a cumulative total of 277 isolates archived between 2010 and 2012 from the Greater Accra Region-Ghana, using the disc diffusion method. The recommendations of the Clinical and Laboratory Standards Institute were used to interpret our results. Resistance patterns were high for co-trimoxazole 232/241 (96.3%), trimethoprim 265/276 (96.0%), erythromycin 255/270 (94.4%), and were low for azithromycin 0/11 (0%), ciprofloxacin 1/274 (0.4%), doxycycline 40/235 (14.5%) and tetracycline 43/232 (15.6%). There was significant increase in antibiotic resistance rates across the year groups studied, except for ciprofloxacin (P =0.5089), trimethoprim (P =0.0533) and erythromycin (P=0.3200). High levels of antibiotic resistance among the present population of *V. cholerae* O1 isolates were observed. However, during cholera outbreaks, azithromycin, ciprofloxacin, doxycycline and tetracycline are alternatives in the treatment and control of infection when not contra-indicated.

Keywords: Ghana: Cholera and drug therapy; *Vibrio cholerae* O1; Drug resistance, microbial; Infection control; Disease outbreak and prevention and control.

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Introduction

Cholera is an infection of the intestine with the bacterium *Vibrio cholerae* O1: El-Tor and Classical biotypes or O139, which produces an enterotoxin. The disease is characterised by acute illness with painless profuse watery diarrhoea with or without vomiting, leading to severe dehydration and death if treatment is not prompt.

One key component in the management and control of cholera, apart from rehydration and hygiene, is antibiotic treatment of cases and contacts, particularly with tetracycline.[1] An efficacious antibiotic helps reduce fluid loss, duration of illness and carriage, severity, fatalities and curbs transmission.[2,3]

According to the World Health Organisation (WHO), cholera continues to remain a major public health problem in the world, especially in Africa and Asia,[4] and without proper diagnosis and treatment, the disease is likely to be imported into non-endemic areas.[5]

Global reports suggests that over 3.5 million people are affected annually with between 100,000-130,000 deaths per year.[6] In recent years, there has been a dramatic increase in the number of notified cholera cases and almost all developing countries are facing either a cholera outbreak or the threat of an epidemic.[4] Between 2nd January and 25th Jun 2006, 1869 suspected cholera cases were reported in Ghana with 79 deaths, a case fatality rate (CFR) of 4.2%. By the end of 2006, the count was 3357 cases and 107 deaths, with an overall CFR of 3.19%.[7] In 2011 and 2012, the Greater Accra Region (GAR) of Ghana recorded 9174 and 6882 cases with 72 deaths (CFR 0.8%), and 48 deaths (CFR 0.7%), respectively.

The contribution of appropriate or inappropriate use of antibiotics in the management, treatment and deaths due to cholera infection has not been clearly distinguished. However, there are copiously documented and well reported evidence of antibiotic resistance by most strains of bacteria worldwide,[8,9] and by *Vibrio cholerae* in particular.[10,11,12,13] Inappropriate use of antibiotics has been implicated as one of the reasons for the high CFR (>5%) in the 2008-2009 cholera epidemic in Zimbabwe.[14] The consequences of using wrong or ineffective antibiotics are therefore dire and cannot be glossed over.

Generally, antimicrobials in the following groups; tetracyclines, fluoroquinolones, macrolides and co-trimoxazole, have been recommended for the treatment of cholera by the Centers for Disease Control and Prevention [CDC],[15] but the choice of specific antibiotics must be informed by local antibiotic susceptibilities. For example, in Ethiopia, recent studies have shown that *V. cholerae* O1 isolates were highly resistant to co-trimoxazole (100%) and less resistant to tetracycline, ciprofloxacin and doxycycline (6.2%, 1.2%, 0%), respectively.[16]

Studies from China also showed varied susceptibility patterns to trimethoprim/sulfamethoxazole (38.5%), tetracycline (11%) and nalidixic acid (45.9%), and strain susceptibilities also varied across different year periods.[13] Similarly, epidemic strains from Haiti have shown increased resistance to nalidixic acid and trimethoprim-sulfamethoxazole.[17] Also, the antibiotic of choice does not only depend on the prevailing effectiveness of the antibiotic against the cholera isolate involved, but a number of factors such as age, sex, and physiological status.

In Ghana, there is scanty information on antibiotic susceptibility profiles of *V. cholerae* O1, and there is no comprehensive and continuous surveillance of epidemic strains of *V. cholerae*. Consequently, the extent of efficacy of current range of antibiotics used in cholera treatment and control cannot be assured. In the current study, we revived and characterized archived epidemic strains of *V. cholerae* O1, determined their resistance profiles to selected and commonly used antimicrobial agents, and assessed differences in resistance patterns across year periods.

Materials and methods

Study design and site

This was a retrospective study in which archived *V. cholerae* O1 isolates, previously obtained from cultured samples at the National Public Health Reference Laboratory (NPHRL), were serotyped and qualitatively screened for antibiotic susceptibilities at the NPHRL over a four month period (April-July

2013). The NPHRL is a body under the Ghana Health Service, and it serves as the reference laboratory for all public health diseases under surveillance, and also coordinates laboratory surveillance activities across the country. The NPHRL also provides support for surveillance activities directly and indirectly to facilities through its satellite branches in Tamale, Takoradi and Kumasi. These satellites serve the northern, western and middle parts of Ghana, respectively. The NPHRL also have additional responsibilities in providing immediate services to inhabitants of the southern and eastern parts of the country, including the Greater Accra, Eastern and Volta regions of Ghana.

Sampling

The NPHRL receives samples (stool or vomitus) of suspected cholera cases from various health facilities in the GAR and beyond. Routinely, upon isolation of any *V. cholerae* O1 bacteria, portions of discrete colonies previously sub-cultured onto a Tryptose soy agar medium were inoculated into a 2ml cryo-tubes, containing 20% glycerol in Tryptose soy broth and appropriately labelled with date and sample source. This was then incubated overnight at 37°C and later stored in a dedicated freezer at -70°C.

The current study analyzed *Vibrio* isolates archived from January 2010 to December 2012. All archived *Vibrio cholerae* O1 isolates at the NPHRL were included in this study. However, further antibiotic susceptibility testing was performed only on the revived and serotyped confirmed isolates.

Revival and confirmation of Vibrio isolates

Archived *V. cholerae* O1 isolates stored at -70°C in cryo-tubes were allowed to thaw at room temperature. A metal wire loop was then inserted into various parts and depths of the Tryptose broth to increase probability of picking portions of media that will contain an isolate.

Thiosulfate Citrate Bile-Salts Sucrose (TCBS) agar plates were then inoculated and incubated at 37°C for 18-24hrs. Similarly, alkaline peptone water, which is an enrichment medium, was simultaneously inoculated and incubated for 4hrs and further sub-cultured onto TCBS and incubated for 18-24hrs at 35-37°C. The culture plates were then observed for bacterial growth after incubation the following day.

Biochemical and serological testing

Suspected yellow colonies from TCBS (*V. cholerae*) were further inoculated onto Mueller Hinton Agar (MHA) and incubated at 37°C for 18-24hrs.

Discrete colonies were tested against oxidase reagent to observe characteristic purple colour reaction within few seconds. Oxidase positive colonies were then serologically typed with commercial polyvalent and monovalent anti-sera (Difco Laboratories, Detroit, MI) for *V. cholerae* O1 or O139. Control strains of *V. cholerae* O1: El-tor (VC20, NICED), Classical (ATCC 11623) and O139 (51394) were included in the analysis.

Antibiotic susceptibility testing

Antibiotic susceptibilities of confirmed *V. cholerae* isolates to selected antimicrobial agents were determined by the disc diffusion method as described by Bauer *et al.*[18] The results were interpreted using the recommendations of the Clinical and Laboratory Standard Institute (CLSI).[19]

Pure colonies of fresh isolates on MHA were emulsified in 2ml sterile saline to obtain turbidity comparable to 0.5 McFarland standard. A sterile swab was then dipped into the inoculum tube and rotated against the side of the tube above the fluid using firm pressure to remove excess fluid. The dried surfaces of MH agar plates were then swabbed in three directions over the entire agar surface; rotating the plate approximately 60 degrees each time to ensure an even confluent distribution of the inoculum. A multi-disk dispenser was loaded with the appropriate antibiotic discs and used to dispense the antibiotic discs onto the MH agar plate on a flat surface, between 3-5 minutes after the streak but not more than 15 minutes and then incubated at 37°C overnight (18-24hrs).

The diameter of zone of inhibition were measured using a caliper and the sizes compared to a standard chart obtained from CLSI to determine susceptibility i.e. whether susceptible (S), resistant (R) or intermediate (I). A control strain of *Escherichia coli* (*E. coli*, ATTC 25922) obtained from the Microbiology Department of University of Ghana Medical School was included in the assay. The antibiotic discs and concentrations used were: erythromycin (15 µg), doxycycline (25 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), co-

trimoxazole (25 µg), azithromycin (30 µg), nalidixic acid (30 µg), streptomycin (10 µg), tetracycline (30 µg) and trimethoprim (25 µg) all from Oxoid (Maryland, MA, USA). Intermediate results were finally classified as resistant, for the purposes of analysis.

Statistical analysis

EPI-INFO 2005 statistical software package (Centers for Disease Control and Prevention, Atlanta, Ga., USA) was used for the entry of all data. Proportions were estimated and statistical differences of resistance patterns across year periods were determined using chi-square and fisher exact tests. Intermediate susceptibilities were grouped as resistant for analysis. P<0.05 was considered significant at 95% confidence level.

Ethical issues

Permission was sought from the NPHRL to use their archived isolates and the study proposal was reviewed and approved by the Ethical Review Board of the Ghana Health Service (Ethical clearance ID No. GHSERC: 16/01/13).

Results

A cumulative total of 277 archived isolates stored from 2010 to 2012 were confirmed as *V. cholerae* O1 and screened against ten different antimicrobials (Table I). Of the 277 isolates, 89 showed resistance to six (6) or more of the ten antimicrobials tested. None of the isolates tested against azithromycin showed any resistance 0/11 (0.0%), whilst resistance levels for ciprofloxacin was as low as 1/277 (0.4%). Isolates tested against co-trimoxazole and trimethoprim showed the highest levels of resistance, 232/241 (96.3%) and 265/276 (96.0%), respectively.

In 2010, the highest resistance patterns were in erythromycin and trimethoprim, 32/33 (97.0%) each, whilst the lowest were in ciprofloxacin and nalidixic acid, 0/33 (0.0%) and 3/33 (9.1%), respectively (Table II). In 2011, resistances were highest in erythromycin and co-trimoxazole, 99/103 (96.1) and 76/81 (93.8), respectively. Ciprofloxacin and nalidixic acid maintained lowest patterns with 1/107 (0.9%) and 13/109 (11.9%), respectively. The patterns in 2012 showed highest resistance in co-trimoxazole, 127/128 (99.2%) and trimethoprim 132/134 (98.5%) and lowest in ciprofloxacin and azithromycin 0/134 (0.0%) and 0/11 (0.0%), respectively.

There was a significant increase in the resistance pattern for nalidixic acid from the levels of 11.9% (13/109) in 2011 to 79.9% (107/134) in 2012 (P< 0.0001). The patterns of resistance for all the antibiotics had significant associations with year of *V. cholerae* O1 isolate across the three years, except for ciprofloxacin (P= 0.5089), erythromycin (P= 0.3200) and trimethoprim (P= 0.0533) (Table II).

Discussion

In the current study, the resistance patterns of antimicrobials tested varied significantly across the year periods of isolates (2010-2012) except for

Table I. Cumulative antibiotic resistance profiles of *V. cholerae* O1 (n=277) for all years (2010-2012), Greater Accra Region, Ghana

Antimicrobial agent	Isolates Tested	No.Resistant (%)
Chloramphenicol	276	184 (66.7)
Nalidixic acid	276	123 (44.6)
Streptomycin	274	245 (89.4)
Tetracyline	275	43 (15.6)
Ciprofloxacin	274	1 (0.4)
Doxycyline	275	40 (14.5)
Erythromycn	270	255 (94.4)
Trimethoprim	276	265 (96.0)
Co-trimoxazole	241	232 (96.3)
Azithromycin	11	0 (0.0)

Table II. Association between antibiotic resistance patterns of *V. cholerae* O1 and year of isolate in the Greater Accra Region, 2010-2012

Antimicrobial agent	2010 n (%)	2011 n (%)	2012 n (%)	X^2	P-value
Chloramphenicol	24/33 (72.7)	63/110 (57.3)	79/110 (71.8)	6.01	<0.0495
Nalidixic acid	3/33 (9.1)	13/109 (11.9)	86/111 (77.5)	113.59*	<0.00001
Streptomycin	26/33 (78.8)	94/108 (87.0)	104/110 (94.5)	7.53	<0.0232
Tetracycline	7/33 (21.2)	26/109 (23.9)	7/110 (6.4)	60.27	<0.00001
Ciprofloxacin	0/33 (0.0)	1/107 (0.9)	0/111 (0.0)	1.35*	0.5089[b]
Doxycycline	5/33 (15.2)	24/109 (23.5)	8/110 (7.3)	9.51	<0.0086
Erythromycin	32/33 (97.0)	99/103 (96.1)	102/111 (91.9)	2.28*	0.3200[b]
Trimethoprim	32/33 (97.0)	104/112 (92.9)	110/111 (99.1)	5.86*	0.0533[b]
Co-trimoxazole	26/29 (89.7)	76/81 (93.8)	105/105 (100.0)	8.38*	<0.0151
Azithromycin	a	a	0/11 (0.00)	N/A	N/A

n (%) number resistant and percentage, X^2: Chi square test.
* Fisher exact test used for cells with less than 5 counts
a No test. N/A: Not applicable, [b]: Not significant

ciprofloxacin, erythromycin and trimethoprim. We also observed multiple drug resistance amongst the *V. cholerae* O1 isolates, with some being resistant to 6 or more of the 10 antimicrobials tested. Even though antibiotics cannot be solely used for the treatment of cholera, it has the added advantage when combined with rehydration therapy in lessening duration of illness, shedding of *V. cholerae* in stools and reducing fatalities by about 50%.[20,21] The timely treatment of cholera patients and contacts with the most effective antibiotics, taking into consideration other contra-indications, requires knowledge of the range of effective antibiotics locally available and applicable within any period.

The drugs recommended by the Ghana Health Service (GHS)[22] for the treatment of cholera are tetracycline, azithromycin, erythromycin, doxycycline and chloramphenicol. However, the current study identified high levels of resistance in erythromycin (94.4%) and chloramphenicol (66.7%), therefore making them unsafe for current cholera management in Ghana. In a pilot study using 27 *V. cholerae* O1 isolates from the 2006 cholera outbreak in Accra, some of the isolates were resistant to tetracycline, trimethoprim and other conventional antibiotics used for cholera treatment.[23] Anecdotally, in a cholera outbreak that occurred between March-July, 2012, in Atebubu-Amanten, a district in the Brong Ahafo Region of Ghana, isolates showed resistance and intermediate susceptibilities to all tested antimicrobials (ampicillin, chloramphenicol, erythromycin, tetracycline, nalidixic acid, co-trimoxazole: sulfamethoxazole trimethoprime), except ciprofloxacin. Increasingly, *V. cholerae* strains are acquiring resistance towards several common antibiotics, posing a great challenge to health care delivery.[24] Tetracycline and quinolones have been widely used to reduce the symptoms of cholera,[25,26] but the increasing advent of multiple drug resistant strains of *V. cholerae* presents enormous challenges to cholera management and require continuous surveillance.[27]

Our observation of erythromycin resistance rates is in variance with findings by Roy *et al.*,[28] that found erythromycin to be the most effective drug for cholera treatment, especially in children. The current study

establishes low levels of resistance to tetracycline (15.6%), ciprofloxacin (0.4%), doxycycline (14.5%) and azithromycin (0%) and therefore provides a better assurance in its usage for treating cholera. A limitation of our study was that only eleven azithromycin antibiotic discs were available for testing. It is therefore a bit premature to conclude that azithromycin is hundred percent effective against our collection of *Vibrio*. Several studies however support the fact that epidemic *Vibrio* isolates are general susceptible to azithromycin, with susceptibility levels ranging from 95 - 100%.[29,30]

Our current findings compare favourably with studies elsewhere that reported low levels of resistance to tetracycline (6.2%), ciprofloxacin (1.2%) and doxycycline (0%) but 100% resistance levels to co-trimoxazole.[1] Co-trimoxazole resistance levels in the current study is however 96.3%. The high rates of resistance to nalidixic acid (44.6%), streptomycin (89.4%), co-trimoxazole (96.3%) and trimethoprim (96%) in the current study is similar to the rates found amongst *V. cholerae* isolates in India.[31] Wang *et al.*,[13] also described similar patterns of resistance to tetracycline (11%) and nalidixic acid (45.9%), but contrasting results for co-trimoxazole (38.5%), when compared to the current study. The stepwise increase and high resistance levels of *V. cholerae* O1 to nalidixic acid between 2010 and 2012 in Ghana has previously been observed in the 2010 Haitian cholera outbreak.[17] This phenomenon is alarming because, antibiotic resistance in nalidixic acid easily spreads to other fluoroquinolones such as ciprofloxacin.

The use of antibiotics alongside rehydration is important as it provides potential means of curbing duration of illness, the shedding of infectious doses via voluminous diarrhoea and further spread of infection. This is particularly crucial in Africa and other developing countries where there is the need to cut down on wastage of otherwise limited hospital consumables such as oral and intravenous fluids to maintain hydration in an environment where access to safe drinking water and rehydration solutions are limited and in acute supply.

Conclusion and recommendations

The current study highlights the increasing high levels of antibiotic and indeed multiple drug resistance amongst clinical isolates of *V. cholerae* O1 in the GAR, including some of those recommended for treatment in Ghana, and also the significant changes in resistance patterns over the years. The high rates of resistance especially to erythromycin is worrisome as it is the drug of choice for pregnant women and children because of the potential side effects from the use of the other available drugs. However, tetracycline, doxycycline, ciprofloxacin and azithromycin remain highly efficacious for the treatment of cholera. These antimicrobials must be prudently used by controlling and limiting their use to only patients with moderate to severe dehydration,[17] to avoid the potential of further spread of resistance. The observed multiple drug resistant epidemic strains of *V. cholerae* O1, and the changing resistance patterns across year periods underscore the need for a continuous surveillance of commonly used antimicrobial agents.

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