Trends in Antibiotic Resistance of Vibrio Cholerae Isolates in Kenya (2006 - 2015)

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Abstract: The evolution of antibiotic resistance was studied among revived *Vibrio cholerae* strains which were previously archived at -80^{0}c between 2006 and 2015. Antibiotic susceptibility testing (AST) on 12 antimicrobials; ampicillin (10μg), cefpodoxime (10 μg), ceftazidime (30 μg), cefotaxime (30 μg), amoxicillin- clavulanic acid (10/ 100 μg ratio) nalidixic acid (30 μg), tetracycline (30 μg), ciprofloxacin (10 μg), SXT (sulphamethoxazole -30 μg trimethoprim -5.2 μg), streptomycin (25 μg), gentamycin (10 μg) and chloramphenicol (30 μg) was carried out using Kirby-Bauer disc diffusion method. AST results revealed susceptibility to tetracycline, which is the drug of choice in Kenya administered as doxycycline during cholera outbreaks, among all isolates. Resistance to βeta-lactams and ciprofloxacin emerged in latter years while a decline in resistance to SXT, Chloramphenicol and Streptomycin was noted. This study gave a clear indication that there were changes in the resistance patterns whereby resistance to some antimicrobials declined and others emerged over the ten year period. In order to slow down the emergence and spread of resistance strains, care should be taken by health professionals when prescribing antimicrobials to patients suffering from cholera disease and should be restricted to only severe cases. It is also recommended that antimicrobial susceptibility testing should be done before giving antimicrobials in management of cholera cases.

Keywords: Antimicrobial resistance, Evolution, Kenya, *Vibrio cholera.*

I. INTRODUCTION

Vibrio cholerae is the causative agent of Cholera disease which has spread globally in seven pandemic waves since 1817 [1]. Though few *V. cholerae* strains are clinically significant to humans, *V. cholerae* type O group 1 is the most important as a cause of epidemic cholera diarrhoea. Cholera causes severe outbreaks of dehydrating diarrhoea which can lead to death if untreated, especially in developing countries thus making cholera an issue of major public health importance in Kenya. This life threatening diarrhoea is attributed to massive luminal secretion of water and electrolytes from enterocytes induced by the cholera toxin [2]. Cholera was first discovered in Kenya in 1971 and since then several outbreaks have been reported thus rating it among the 35 priority diseases in Kenya [3]. Currently, the standard way of treating severe cases of cholera is through administering rehydration fluids. However, the use of antibiotics reduces the duration of diarrhoea by 50 – 56 %, reduces stool output by 8 – 92 % and reduces shedding of bacteria by 26 – 83 %. In general, antibiotics reduce the duration of illness by up to 50 % [4].Despite the advantages associated with the use of antibiotics, it has been compromised by the evolution and spread of strains conferring resistance to multiple antibiotics including those which have been recommended by WHO that include doxycycline, furazolidone, sulfamethoxazole, trimethoprim, chloramphenical and ciprofloxacin. The most common treatment used against bacterial pathogens is the administration of β-lactam antibiotics such as penicillins, monobactams and cephalosporins but the emergence of β-lactamases that mediate resistance to these antibiotics is on the rise [5]). B-lactamases are rare in *Vibrio* but recently, the prevalence of these

enzymes has been increasing in this Genus. Resistance to antibiotics poses a global threat to the fight against infections and has been shown to vary with time in *V. cholerae.* Therefore, it becomes a necessity to study evolution of antibiotic resistance over a period of time. This knowledge will then assist in making decisions with regards to antimicrobial use and also in coming up with strategies of controlling the emergence and spread of resistant strains.

II. METHODS

The study was conducted at Center for Microbiology Research, a unit of the Kenya Medical Research Institute where 130 strains of *Vibrio cholerae* are stored at -80 ^{0}C. The archived strains had been previously isolated from rectal swabs using standard bacteriological methods. The numbers of isolates per year included in this study were as follows 23 (2006), 13 (2007), 11 (2009), 14 (2010), 19 (2012) and 50 (2015).

The archived *V.cholerae* isolates were removed from the -80^0 C freezer and allowed to thaw at room temperature. A loopful of the isolate was inoculated using the streak plate method on Thiosulphate Citrate bile salts Sucrose agar (TCBS, a selective media for *V. cholerae*) at 37 ^{0}C for 18 to 24 h *V. cholerae* were identified as large (2 to 4 mm in diameter) shiny, yellow, opaque colonies. These were then subcultured on Muller Hinton agar and incubated at 37^0 C for 18 to 24 h. A known *V. cholerae* strain was used as positive control and *E.coli* ATCC 25922 strains was used as negative control. Pure colonies were subjected to serological identification methods. This was done using the slide agglutination technique using polyvalent antisera for *V.cholerae* type 01 and monovalent antisera for *V. cholerae* type 01 serotypes Inaba and Ogawa. A well isolated colony was picked and mixed in a drop of sterile normal saline on a glass slide to make a milky suspension. A drop of the antisera was then mixed to check for the presence or absence of agglutination within two minutes. Presence of agglutination confirmed the serotype and subtype of the strains. Positive and negative controls were included.

Kirby-Bauer disk diffusion method was used for susceptibility testing. In order to obtain 0.5 McFarland opacity equivalent suspension of the isolate, one colony was picked from Mueller Hinton agar and suspended in 3 mL of normal saline. Using a sterile cotton swab, a thin smear of this suspension was spread evenly on MH plate to obtain uniform growth upon incubation. Antimicrobial susceptibility testing was performed using commercial discs following manufacturer's instructions. Two plates were used on one plate the following drugs were applied using a dispenser ampicillin (10μg), cefdoxime (10μg), ceftazidime (30μg), and cefotaxime (30μg) then with the use of a sterile cool forceps; amoxicillin- clavulanic acid (10/100μg ratio) was placed at the center of the plate. On the second plate the following drugs were applied nalidixic acid (30μg), tetracycline (30μg), ciprofloxacin (10μg), trimethoprim (5.2μg), streptomycin (25μg), sulphamethoxazole (30μg), gentamicin (10μg) and chloramphenicol (30μg). The control strain was *E.coli* ATCC 25922 which was subjected to the same set of discs as it is virtually susceptible to all antimicrobials and therefore used to ascertain potency of the discs and quality of the media.

III. RESULTS

Apart from tetracycline antimicrobial whose susceptibility was noted among all isolates, resistances were noted for other antimicrobials. Presence of resistance to each antimicrobial among *V.cholerae* isolates was noted between 2006 and 2015 (**Fig 1**).

Resistance to ampicillin was noted in 2006 in very few isolates (4%) and 7% of isolates from the 2010 period were resistant to this antimicrobial. However, none of the isolates obtained in 2007 and 2009 were resistant to ampicillin. The prevalence of such strains continued to rise by 77 % in 2012 and a further 2 % in 2015. The reason behind the high number of isolates resistant to ampicillin in 2012 and 2015 as compared to other years (p: <0.001 OR: 0.0057, CI: 0.0012-0.0274) is still not clear. Resistance to amoxicillin/clavulanic acid (amoxyclav) varied over the years. For instance, in 2006, 74% of isolates were resistant to amoxyclav but this declined by 28% in 2007 with the numbers declining even further by 36% in 2009 (p: 0.045, OR: 17.69, CI: 2.02-154.2). However, in 2010, the number of isolates resistant to amoxyclav increased by 6% and this increased by 65% in 2012 with 2015 reporting the highest number of isolates (90%) resistant to this amoxyclav.Isolates obtained in 2006, 2007 and 2009 did not exhibit resistance to third generation cephalosporins (cefotaxime, cefpodoxime, ceftazidime) (**Fig 1).**
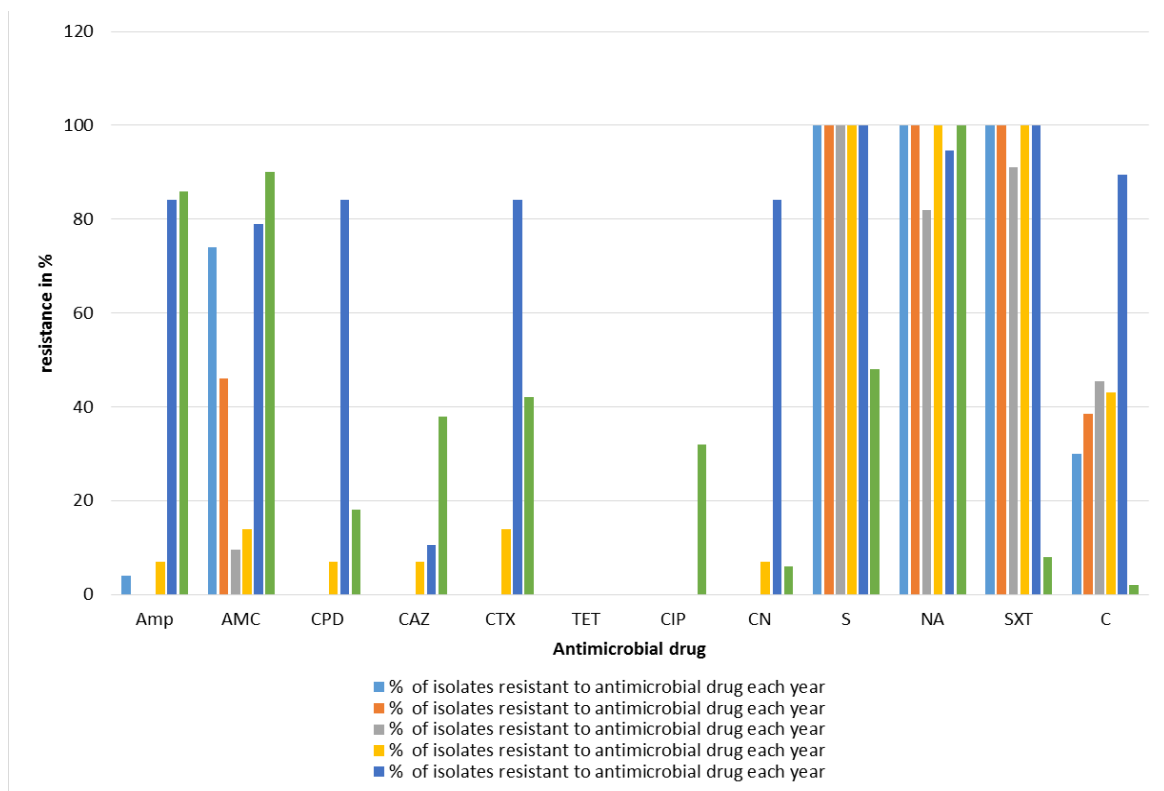
Fig 1: Trends in resistance to various antimicrobials between 2006 and 2015

The first isolates resistant to cefpodoxime and ceftazidime emerged in 2010 and these accounted for 7% of all isolates obtained in this year. In the same year, 14% of isolates were resistant to cefotaxime. Resistance to these third generation cephalosporin continued in 2012 where it increased by 70% for cefotaxime and cefpodoxime. Resistance to ceftazidime continued to rise by 28 % from 10.5% in 2012 to 38% in 2015. This was however not the case for the other two third generation cephalosporins (cefotaxime, cefpodoxime) where a decline in resistance of up to 62% was noted for cefotaxime and a decline of 66% noted for cefpodoxime from 2012 to 2015. This decline could be a result of absence of isolates producing ESBLs phenotypically though this remains an assumption.

Resistance among the quinolones ,that is, ciprofloxacin and nalidixic acid varied among the isolates (**Fig 1).** Isolates collected in 2006 ,2007,2009,2010 and 2012 were susceptible to ciprofloxacin which is a broad spectrum fluoroquinolone. However 34% of isolates obtained in 2005 had intermediate resistance to this antimicrobial. On the other hand, all isolates in 2006 and 2007 were resistant to nalidixic acid which was followed by a decrease of 18% to 82% in 2009. This difference was however not statistically significant ($p: < 0.199$, CI: 0.9-16). All isolates in the ten year period reported resistance of not less than 80% to nalidixic acid indicating that *V.cholerae* isolates are highly resistant to nalidixic acid and this resistant has persisted over time.

Resistance patterns against the aminoglycosides, gentamicin and streptomycin varied over the ten year period (**Fig 1**). None of the isolates obtained between 2006 and 2009 were resistant to gentamicin as compared to 7% of isolates obtained in 2010. Resistance to this aminoglycoside increased by 77% in 2012. The reason behind this sharp increase is still not clear though this was the same year when isolates producing ESBLs emerged. This was however different for streptomycin whereby all isolates obtained between 2006 and 2012 2006 were resistant to this antimicrobial. There was however a decline in resistance in 2015 by 52% ($p<0.001$).

Isolates in 2006 and 2007 were all resistant to SXT (**Fig 1**). It is still not clear how the resistance pattern progressed in 2008 but in 2009, a decrease to 91% from 100% in resistance was noted. This resistance then increased to 100% in 2010 and persisted in 2012. However, a significant increase in drop from 100% in 2012 to 8% in 2015 was noted ($p: <0.001$, OR: 184.3, CI: 23.4-1450).

Thirty (30 %) of isolates in 2006 were resistant to chloramphenicol (**Fig 1**). This resistance increased steadily in 2007 by 8% through to 2009 by 7%. The number of isolates resistant to this antimicrobial increased from 30% in 2006 to 38.5% in 2007 then 40% in 2009 and 2010. A sharp increase from 43% in 2010 to 89% in 2012 thus reporting the highest number of isolates resistant to chloramphenicol over the ten year period (p: <0.001, OR: 0.0325, CI: 0.07-0.1504). This was the same year when isolates producing ESBLs emerged but it is still not clear if resistance to chloramphenicol is linked to the ESBL phenotype. The year 2015 reported the least number of isolates resistant to chloramphenicol where a decrease from 89.5% in 2012 to 2 % in 2015 was noted (p: <0.001, OR: 49, CI: 6.44 – 372)

IV. DISCUSSION

Antimicrobial susceptibility revealed that majority of these isolates (>50%) collected over the ten-year period were resistant to multiple classes of antimicrobials. Recent studies carried out between 2007 and 2009 in Kenya also revealed that resistance to multiple antimicrobials including those recommended by world health organization (WHO) namely chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim [7]. In this study, highest prevalence of resistance of more than 90% was recorded against nalidixic acid as compared to other antimicrobials among all isolates analyzed regardless of year of isolation. The most effective antimicrobial was found to be tetracycline followed by ciprofloxacin that was effective to more than 70% of total isolates. These data therefore indicate that tetracycline still remains an antimicrobial of choice in the management of cholera disease. Among these strains, the most resistant (MDR) were those obtained in year 2012. Taken together this data suggest that resistance to antibiotics is on the increase among *V. cholerae* strains [8]. These epidemics of MDR cholera have been reported worldwide and have been associated to indiscriminate use of antibiotics during treatment of cholera and other enteric diseases.

Resistance to nalidixic acid among *V.cholerae* has been reported over a period of time and this finding was confirmed in this study. However, intermediate resistance to ciprofloxacin, broad spectrum quinolone, which has been a very effective antimicrobial against *V.cholerae,* emerged in 2015 and has not been reported in past isolates obtained locally. On a broader perspective, resistance to nalidixic acid remained constantly high (> 80%) among all isolates collected over the ten year period indicating that this antimicrobial is no longer reliable in the management of cholera cases in Kenya. On the other hand, ciprofloxacin showed 100% effectiveness (zone diameters of above 25mm) in more than 90% of *V.cholerae* isolates collected between 2006 and 2012. This is in agreement with other findings that concluded ciprofloxacin to be an effective antimicrobial in the management of cholera cases [9]. However, isolates showing intermediate resistance to this antimicrobial were noted in 2015 indicating a possibility of resistant strains emerging in the near future. Although the factors behind emergence of these resistances are yet to be determined, such resistances could have emerged as a direct response to strong selective pressure exerted by nalidixic acid in clinical setups. Related studies indicate that use of basic quinolones such as nalidixic acid can exert a selective pressure that may favour the emergence of resistance to advanced classes such as fluoroquinolones. Though mechanism of resistance to quinolones could not be established in this study, it has been associated with spontaneous mutations in *V. cholerae* chromosome in the *gyr*A genes which encode subunits of DNA gyrase and *Par*C genes which encode subunits of topoisemerase IV [9] ,[10]. Similar strains which were resistant to quinolones were reported in isolates collected between 2007 and 2010, resistance to nalidixic acid was at 84% while resistance to ciprofloxacin was at 2.3%. Similar strains have also been reported in Zimbabwe, Nigeria and Cameroon [11]. This emergence of *V.cholerae* strains resistant to quinolones, might greatly complicate therapeutic use of antimicrobials putting into consideration that ciprofloxacin is used as second line of treatment to cholera when doxycycline is unavailable. Therefore, early intervention and close monitoring of the susceptibility trends is necessary.

The first isolates (29%) resistant to third generation cephalosporin (cefotaxime, cefpodoxime, ceftazidime) emerged in 2010. This was in agreement with other findings where Kenyan isolates collected between 1994 and 2007 did not exhibit resistance to third generation cephalosporin [7]. The prevalence of such strains continued to rise by more than 70% for cefotaxime and cefpodoxime in 2012 but this prevalence declined to 10% in 2015. Presence of immigrants to Dadaab refugee camps from neighbouring countries like Somalia which was experiencing a serious outbreak could have contributed in the dissemination of these isolates [12]. These strains also expressed high resistance to aminoglycosides (streptomycin, gentamicin) and chloramphenicol which is in agreement with other findings which have associated production of extended specum β lactamases with resistance to other classes of antimicrobials [13].

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V. CONCLUSION

Considering antimicrobials are not recommended for cholera, this resistance could be occurring as a result of acquisition of resistance genes from other Enterobacteriaceae given that these organisms interact in the same environment.

Tetracycline was the most potent antimicrobial, however, resistance is likely to emerge if its use is not limited and this will then further complicate management of cholera disease in the country considering it is the first line treatment in management of cholera. Increase in susceptibility to some antimicrobials which were previously resistant like SXT, streptomycin and chloramphenicol was noted thus giving an indication that resistance to antimicrobials can actually be controlled. Following trends in the resistance patterns of commonly used antimicrobials like β-lactams, third generation cephalosporin and ciprofloxacin, there stands a risk of returning to the pre-antimicrobial era. Emergence of extended specum β lactamases is increasingly becoming complex among *V.cholerae* isolates and this is likely to create therapeutic problems in future thus timely detection of these isolates is of great importance. Given that the resistance to antimicrobials is on the increase, it is now prudent to consider vaccinating vulnerable populations against cholera.

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