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Emerging quinolones resistant transfer genes among gram-negative bacteria, isolated from faeces of HIV/AIDS patients attending some Clinics and Hospitals in the City of Benin, Edo State, Nigeria

Authors
Enabulele IO
Department of Microbiology, University of Benin, Benin City, Nigeria
Yah SC
Department of Medical Microbiology, and Parasitology, Madonna University, Okija, Nigeria
Yusuf EO
Department of Microbiology, University of Benin, Benin City, Nigeria
Eghafona NO
Department of Microbiology, University of Benin, Benin City, Nigeria

Address For Correspondence
Yah Clarence
Dept of Medical Microbiology & Parasitology,
Madonna University, Okija,
Anambra, State, Nigeria.
E-mail: yahclar@yahoo.com

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Abstract:
A survey of 1431 gram-negative bacilli from June 2001 to September 2005 were obtained from the faeces of 920 HIV/AIDS patients attending some Clinics and Hospitals in Benin City, Nigeria, were screened for quinolones resistance gene. The HIV/AIDS patients CD4 cells range was ≤14/mm^3 ≥800/mm^3 of blood. Out of the 1431 isolates, 343 (23.9%) were resistance to quinolones with a MIC ≥4µg/ml for norfloxacin, ciprofloxacin and pefloxacin while a MIC of ≥32 µg/ml for nalidixic acid. The screened isolates include *Pseudomonas aeruginosa* 64(18.7%), *E coli* 92(26.8%), *Klebsiella pneumoniae* 53(15.4%), *Salmonella typhi* 39(11.4%), *Shigella dysenteriae* 36(10.5%), *Proteus mirabilis* 34(9.9%) and *Serratia marcescens* 25(7.3%). The average resistance of the isolates to the various quinolones ranged from 42.7% to 66.7%. *Klebsiella* were the most resistant isolates with a mean resistance of 66.7% while *Proteus* were the less resistant isolates with a mean resistance of 42.7%. Most isolates were resistant to Nalidixic acid followed by norfloxacin while the less resistant were to the pefloxacin. The frequency of qnr genes transfer to EJRif^r as recipient ranged from 2 x 10^{-2} to 6 x 10^{-6} with an average of 2 plasmids per cell. The molecular weight of the plasmids ranged from ≤2.9kbp to ≤5.5 kbp. This indicated that plasmids allowed the movement of genetic materials including qnr resistant genes between bacteria species and genera in Benin City, Nigeria.
Key Words: Resistance Gene Transfer, HIV/AIDS, Gram-negative bacilli

Introduction:
The remarkable success of antimicrobial drugs generated a misconception in the late 1960s and early 1970s that infectious diseases had been conquered. However, 40 years later, infectious diseases remain the third-leading cause of death, both in the third world and the developed countries and are the second-leading cause of death worldwide.(1) Furthermore, the emergence of multi-drug-resistant bacteria has created a situation in which there are few or no treatment options for infections with certain microorganisms.(1) The advent of bioterrorism, which gained widespread public attention after 11, September 2001, has magnified the problem, because genetic engineering of pathogens could render them resistant to currently available antimicrobials.(2,3)

The human immunodeficiency virus (HIV) infected persons have an exceptional vulnerability to invasive bacterial infections that is much greater than that seen in immunocompetent, HIV-uninfected persons. There are numerous defects in the immune system that tend to affect the human body by destroying the host defense mechanisms, responsible for the immune deficiency.(4-8) Therefore reducing these immune cells in the body and shutting off the immune response network increase the vulnerability of HIV-infected person to serious bacterial illness and several forms of tumors. At this point, the defense cells are lowered and the patient develops full-blown AIDS or succession of illnesses and dies.(1,8-10) These deaths were primarily due to pulmonary infections, diarrhea, and malnutrition.(11) These include defects in the cell-mediated (T cell) and the humoral (B cell) arms of the immune system; phagocytic abnormalities including decreases in neutrophil number, multiple defects in neutrophil function, and impairment in macrophage and monocyte function;(12) and defects in three components of complement.(13). These defects become more severe as the person's HIV disease progresses. Venerable factors in developing countries that increase susceptibility to infection in HIV/AIDS-infected person include frequent use of common broad-spectrum quinolones, malnutrition, micronutrient deficiencies, and lack of adequate medical care, frequent hospitalizations, and the use of indwelling intravascular catheters that disrupt the integrity of the skin.(14). This increases the intestinal colonization rates among the enteric pathogens and recurrent infections with the same bacterial species. Bacteria associated with gastroenteritis in HIV/AIDS-infected patients include *Salmonella* species, *Shigella* species, *Campylobacter* species, *Aeromonas hydrophilia,* enterotoxigenic, enterohemorrhagic, enteropathogenic or enteroinvasive *E coli, Vibrio* species, and *Clostridium difficile*. *Salmonella* species, *Klebsiella* species, *Enterobacter* species, *Enterococcus* species, *Pseudomonas* species, *Proteus* species, *Morganella* species, *Pseudomonas aeruginosa, Shigella* species, and *Campylobacter* species may

disseminate and cause wide spread serious infection. The presence of white blood cells, blood, parasites, and *Clostridium difficile* toxin in stool samples should be established, and bacterial culture should be performed. Special tests must be performed to determine the presence of disease-causing *E coli*. If the infection is due to *Salmonella* species or *Shigella* species treatment with ampicillin, TMP/SMX, cefotaxime, or ceftriaxone should be initiated. Treatment for *Campylobacter* species infection includes erythromycin or azithromycin. Bacterial isolates should be tested for antibiotic susceptibility and the antibiotic regimen should be adjusted accordingly. Several reports have described quinolones resistance among pathogenic bacteria (15-17) and have documented a number of treatment failures associated with pathogens showing decreased susceptibility to commonly prescribed quinolone agents.(15,18)

The aims of the present study were to investigate the multiple quinolones resistant pathogens from HIV/AIDS patients in Benin City in relation to their plasmids profile. This is because our knowledge on the prevalence and diversity of quinolones plasmids mediated bacteria is very limited. Such information will be of use to augment the present knowledge on drug resistance in our community by providing information on the appropriate choice of antimicrobial agents and the factors that aggravate the drug-resistance problem in our society.

Materials and Methods:

A survey of 1431 gram-negative bacilli from June 2001 to September 2005 were obtained from the faeces of 920 HIV/AIDS patients attending some Clinics and Hospitals in Benin City, Nigeria. The patients were screened for their human Immunodeficiency virus (HIV) antibodies using the Wellcozyme ELISA technique as recommended by Wellcome while the cytometer was used for the CD4 cells count (Partec Cyflow Germany).

The faeces samples obtained were inoculated aerobically on sterile blood agar, MacConkey agar, Cystine lactose electrolyte deficient agar, eosin methylene blue, nutrient agar and nutrient broth, triple sugar iron agar and *Salmonella-Shigella* agar (19) at 37^0C for 24 hours. The colonies of each representative isolates were then characterized using standard bacteriological method according to Cowan and Steel.(20) Other tests included gram staining, pigment production, hemolysin production, motility, indole, urea, citrate utilization and hydrogen sulfide production, oxidase, and sugar fermentation were used to isolate the enteric gram negative bacteria. They were further sub cultured on nutrient agar slants and stored at 4^0C for further analysis.

Antibiotics Susceptibility Testing

Susceptibility testing was determined both by overnight broth-micro-dilution (Etest) and agar disk diffusion methods as recommended by Bauer *et al* (21) and National Committee for Clinical Laboratory Standard (NCCLS) (22) using Oxoid- Mueller Hinton agar plates (Difco Laboratories, Detroit, Mich). The following antibiotics were used to screen for the resistance of the isolates; ciprofloxacin (CIP) 10µg, Nalidixic acid (NA) 10µg, Norfloxacin (NB) 10µg and Pefloxacin (PEF) 10µg. The zones of inhibition were then measured and the results recorded as sensitive (s) or resistant (r) based on World Health Organization Drug Information (23) and National Committee for Clinical Laboratory Standard.(22)

Etest MIC Agar Dilution tests

The MICs of the quinolones were determined using the Etest system (AB-Biodisk, Solna, Sweden) according to manufacturer instructions. The plates were inoculated by swabbing the surfaces with a 0.5 McFarland standard gram-negative bacterial isolates suspension using Mueller Hinton agar plates ((Difco Laboratories, Detroit, Mich). The Etest strips were applied onto the surface of the inoculated Mueller Hinton agar medium and the plates were then incubated at 35^0C-37^0C for 24 hours. After 24 hours of incubation, elliptical zones of inhibition of bacterial growth were seen around some of the test strips. The zone edge intersects of the plastic strip were read as the specific level corresponding to the inhibitory concentration of the drug that inhibits the microorganism. The minimum inhibition concentration (MIC) were then measured and the results recorded as sensitive (s) or resistant (r) based on World Health Organization Drug Information (23) and

National Committee for Clinical Laboratory Standard –NCCLS.(22)

Transfer experiments were carried out by the liquid method technique.

Conjugation and Plasmids profiles

Conjugations were performed using *E coli* strains (EJRif^r) obtained from Nigerian Institute for Medical Research (NIMR) as recipient as previously described by Olukoya and Oni.(24) The donors and recipients-plasmid-free-rifampicin resistant strains were incubated both on Luria broth culture (Difco Laboratories, Detroit, MI) at 37^{0}C for 18 hours. The transconjugants were selected on nutrient agar (Nutrient agar-International Diagnostic Group UK) medium supplemented with the correspondence antibiotics and rifampicin 200µg/ml to inhibit the growth of the donor and recipient respectively. Curing was carried out according to Miller (25), Olukoya and Oni.(24)

The transconjugants were re-streaked onto fresh selective nutrient plates and their identities were re-confirmed on the basis of their biochemical methods and their antibiotics resistance pattern. The Zhou *et al* (26), usually called TENs-mini-prep in ten minutes, method was employed for screening plasmids (rapid alkaline extraction) of donors and transconjugants. The plasmids DNA were then electrophoresed on 0.8% agarose gel, stained with 7µl ethidium bromide. The DNA was then photographed and viewed using UV trans-illuminator. The molecular weights and distances were then determined using standard methods according to Meyers *et al* (27) using standard DNA molecular weight marker II (0.12-23.1kbp) of bacteriophage lambda Hind III (2027, 2322, 4361, 6557, 9416 and 23130) Cat number 236250 (Roche Diagnostic GmbH).

Results:

The results show that out of the 1431 gram-negative bacilli from June 2001 to September 2005 obtained from the faeces of 920 HIV/AIDS patients attending some Clinics and Hospitals in Benin City, Nigeria, the resistant isolates included *Pseudomonas aeruginosa* 64(18.7%), *E coli* 92(26.8%), *Klebsiella pneumoniae* 53(15.4%), *Salmonella typhi* 39(11.4%), *Shigella dysenteriae* 36(10.5%), *Proteus mirabilis* 34(9.9%) and *Serratia marcescens* 25(7.3%) as shown in Table 1.

Table 1: Percentage (%) of gram-negative bacilli with quinolones resistant antibiotics

Organisms	Number of Resistant Isolates	Percentage of Resistant Organisms To Antibiotics				Average Resistant
		NA	NB	CIP	PEF	Quinolone
P. aeruginosa	64(18.7%)	82.8%	43.8%	28.1%	23.4%	44.5%
E. coli	92(26.8%)	54.3%	67.4%	30.4%	22.8%	43.7%
K. pneumoniae	53(15.4%)	84.9%	66.0%	5.5.6%	60.4%	66.7%
S. typhi	39(11.4%)	71.8%	48.7%	51.3%	41.0%	53.2%
S. dysenteriae	36(10.5%)	61.1%	52.7%	41.7%	44.4%	50.0%
P. mirabilis	34(9.9%)	58.9%	41.2%	32.4%	38.2%	42.7%
Serratia marcescens	25(7.3%)	68.0%	52.0%	36.0%	40.0%	49%
Total	343(100%)					

Key: NA = Nalidixic acid, NB = Norfloxacin, CIP = Ciprofloxacin, PEF = Pefloxacin

Table 2: Antibiotic Resistance and Plasmid Profile of gram-negative bacteria Obtained from some Clinics and Hospitals in Benin City Nigeria

Isolates	Resistant spectrum Before transfer	No with Plasmids	Plasmids size (kbp)	Transferred Plasmids size (kbp)	Frequency of Transfer	Resistant Spectrum after Transfer
01	CIP, NA NB	3	2.9, 4.8, 5.5	2.9, 4.8	$6x10^{-4}$	NA,NB
1	CIP, NB,NA	2	3.0, 6.0	3.0, 6.0	$3x10^{-4}$	NA,NB
2	PEF	0	-	-	-	-
3	PEF,CIP, NB	2	2.9, 4.8	2.9	$2x10^{-2}$	NA,CIP
5	NA, NB	2	2.9, 4.8	4.8	$6x10^{-2}$	NA,NB
6	NA,NB	2	3.0, 6.0	3.0, 6.0	$3x10^{-1}$	NA
11	CIP	0	-	-	-	-
15	NA PEF	1	2.9	-	-	-
20	NA,CIP	2	2.9, 4.8	2.9,4.8	$6x10^{-2}$	NA
29	NB, CIP NA	1	3.0	3.0	$2x10^{-2}$	NA,CIP
98	NB, NA, CIP	2	3.0, 6.0	3.0	$3x10^{-4}$	NA,NB,CIP
231	NB,NA	3	3.0, 6.0, 5.5	6.0, 5.5	$6x10^{-2}$	NA,NB
246	NA,NB, PEF	3	2.9, 3.0, 4.8	2.9, 4.8	$6x10^{-2}$	NA,NB
899	NB,CIP	1	3.0	3.0	$6x10^{-2}$	-

Key: 01=*E. coli,* 1=*S. dysenteriae*, 2=*E. coli,* 3=*S. dysenteriae*, 5=*E. coli,* 6=*K. pneumoniae, 11= P. aeruginosa,* 15= *E. coli,* 20=*Serratia marcescens, 29=E. coli 98 = P. aeruginosa*, 231 = *P. mirabilis*, 246 = *S. typhi,* 899 = *S. typhi,* NA = Nalidixic acid, NB = Norfloxacin, CIP = Ciprofloxacin, PEF = Pefloxacin

The HIV/AIDS patients CD4 cells count range was ≥14/mm^3 ≤800/mm^3 of blood. Age group 21-30 years had CD4 cells count range from 102 cells/µl -628 cells/µl (x±SD, 208±145.41), age group 31-40 years with CD4 cells count range from 34 cells/µl-347 cells/µl (x±SD, 125.95±134.30) and greater than 41 years had CD4 cells count range 16 cells/µl-528 cells/µl (x±SD, 102.05±184.95) as shown in Table 3, before commencement of antiretroviral chemotheraphy.

Table 3. CD4 count (cells/ul of blood) obtained from HIV/AIDS patients attending some clinics and hospitals in Benin City, Nigeria

Age group (years)	CD4 count (cells/µl of blood) on positive cases (x±SD)
21-30 (n= 509)	102/µl-628/µl (208±145.41)
31-40 (n=348)	34/µl-347/µl (125.95±134.30)
>41 (n=63)	16/µl-528/µl (102.05±184.95)

Out of the 1431 isolates screened, 343 (23.9%) were resistant to quinolones with a MIC ≥4µg/ml for norfloxacin, ciprofloxacin and pefloxacin while a MIC of ≥32 µg/ml for nalidixic acid. The MIC susceptibility break point for ciprofloxacin was ≤1µg/ml with an intermediate potency of 2µg/ml while the MIC break point for nalidixic acid was ≤8µg/ml with intermediate potency of ≥16µg/ml. The average resistance of the isolates to the various quinolones ranged from 42.7% - 66.7%. *Klebsiella* were the most resistant isolates with a mean resistance of 66.7% while *Proteus* was the less resistant isolates with a mean resistance of 42.7%. Most isolates were resistant to nalidixic acid followed by norfloxacin while the less resistant were to the pefloxacin. The frequency of qnr genes transfer to EJRif^r as recipient ranged from 2 x 10^{-2} - 6 x 10^{-6} with an average of 2 plasmids per cell. Transfer qnr resistant genes were found in 10/14(71.4%) of the transconjugants tested while 4/14(28.6%) was not successfully transferred. The MIC of ciprofloxacin of all the transconjugants was ≥4µg/ml while that of nalidixic acid was ≥16µg/ml. The molecular weights of both the donor and transconjugants of the plasmids ranged from ≤2.9kbp - ≤5.5kbp. The Plasmids bands are shown as in Fig 1.

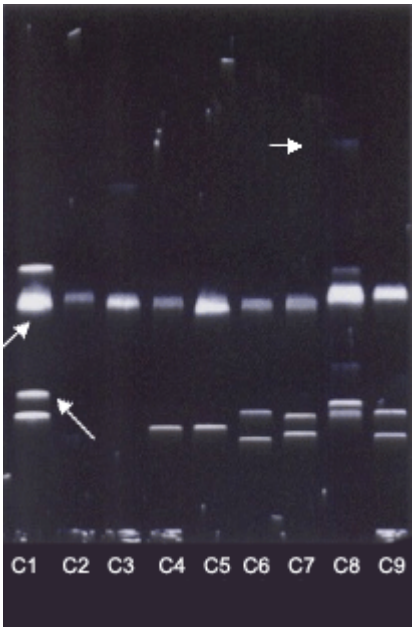

Fig 1: Plasmids DNA bands of some selected isolates from some Clinics and Hospitals in the City of Benin, Edo State, Nigeria. Separation of DNA molecular weight on agarose gel stained with ethidium bromide according Zhou et al. (26-1990). Line C1 standard DNA marker, lines C2, C3, C4, C5, C6, C7, C8, C9 are plasmid DNA bands of test isolates.

This indicated that plasmids allowed the movement of genetic materials including qnr resistance genes between bacteria species and genera in Benin City, Nigeria. Curing experiments showed only the chromosomal DNA with molecular weight ≥23.1kb without the plasmids after using Birnboim and Doly method.(28)

Discussion:

Multiple antibiotics resistance to useful classes of antibiotics including beta lactams, aminoglycosides and quinolones have generally increased among a number of gram-negative hospital pathogens from HIV/AIDS patients. Life threatening bacterial infections have occurred in patients who become immunocompromised after chemotherapy for cancer or HIV/AIDS or immunosuppressive therapy for organ transplantation.(29,30)

With the HIV/AIDS pandemic this has lead to an increase in the resistance rate. Although quinolone resistance commonly results from chromosomal mutation, recent studies indicate that such resistance can also be transferred on plasmids carrying the quinolone resistance gene (*qnr)* in Benin City, Edo State, Nigeria.

Inappropriate use of antibiotics is known to play a major role in the development and spread of resistant bacteria. Among the isolates, *Klebsiella pneumoniae* was 84.9% resistant to nalidixic acid, followed by *Pseudomonas aeruginosa* (82.8%) and *Escherichia coli* (54.3%), the least resistant isolates to nalidixic acid. These correspond with the results of Enabulele *et al* (31), Yah *et al* (32) and Yah *et al* (16) where they consecutively found that among the quinolones, nalidixic acid was the least sensitive quinolones to the isolates from burns wounds and bacteriology of kerosene burn wounds respectively from the University of Benin Teaching Hospital accident emergency ward. This reflected the idea that nalidixic acid and norfloxacin are the oldest and most abused/misused quinolones as compared to the newer and most potent ciprofloxacin and pefloxacin. The study also showed a high diversity of plasmids among the quinolones resistance isolates. These observations appear high compared to those Obasiki-Ebor and Salami (33), Enabulele *et al* (31). The results of the current study were in accordance with those of Wang *et al* (15) while detecting emerging of plasmid-mediated quinolone resistance associated with the quinolone gene in *Klebsiella pneumoniae* in clinical isolates in the United States, confirmed the diversity of plasmid transfer among isolates. The results also indicated that quinolones resistance plasmids carried by Nigerian clinical isolates lack discernible evolutionary lineages, instead demonstrating the distribution of similar resistance profiles in diverse genetic backgrounds. The sizes of the various plasmids ranged from 2.9kbp to 6.0kbp. The fact that these plasmids were not similar in their sizes and resistance markers showed that there was no plasmid epidemic involved in the antibiotic resistance of the isolates or such incidence reported in the community of study. The number of plasmid bands per isolate did not reflect the nature of resistant markers. The plasmids' molecular weights obtained in this work were very smaller in size as compared to plasmids isolated by other workers with very large molecular weight plasmids.(31,34,35) According to Aluyi and Akortha(35), these multiple copies of plasmid bands might result from covalently

close circular, open circular and linear forms of the same plasmid that migrated at different rates on agarose gel electrophoresis.

Other findings by Enabulele *et al* (31) also found that a large majority of *Klebsiella pneumoniae* from University of Benin Teaching Hospital (UBTH) in Nigeria, harbour plasmids as compared to other isolates. Ling *et al* (36) also found that *Klebsiella* species up to 17% from Beijing and Shanghai hospitals harbour the highest rate of resistance β-lactams antibiotics. Ling *et al* (36) concluded that these differences were backed by prescription policies in the various health centers. Sheikh *et al* (37) in Pakistan found that plasmid borne antibiotics resistance factors among indigenous *Klebsiella* can be transferred from *Klebsiella* to *Escherichia coli* MD40 (recipient). They also discovered that some of the plasmid borne resistance markers were non-conjugative/non-transferable but a tremendous conjugative plasmids were found to carry potentials to disseminate resistance markers to distant recipient cells. In many developing countries, well-trained health personnel are scarce and cannot serve the entire population, especially in rural areas. Community health workers and others with minimal training treat minor ailments.(38,39) The qualifications and training of community health workers, as well as the quality of care they provide, vary from country to country. Unskilled personnel are less aware of the deleterious effects of inappropriate antibiotic use. In Thailand for example, pharmacy technicians prescribed rifampicin for urethritis and tetracycline for young children.(40) Unqualified drug sellers offer alternative drugs when the prescribed drugs are out of stock or refill prescriptions without consulting the prescriber.(41,42) Untrained practitioners treat a high proportion of patients in some developing countries simultaneously with oral and injectable quinolones administered with contaminated needles and syringes (43-45) for misdiagnosed noninfectious diseases.(46)

Within the Sub-Saharan countries including Nigeria the quinolones have always been regarded as the most potent antibiotics. The rate of emerging qnr resistance genes due to mobile genetic elements and chromosomes were found to be at the high side in the study as compared to other results of quinolones resistant 10 years ago. The transfer experiment indicated that resistances of quinolones to the various isolates were both plasmid and chromosomes mediated. Resistance to nalidixic acid and norfloxacin were highly mediated by plasmids as compared to others but chromosomal mediation was on a higher side. The transferred plasmids were identical both in sizes and molecular weights to their donor's resistance plasmids when subjected to electrophoresis. The results predicted a rapid rise/spread in quinolones resistant isolates in the near future as the HIV/AIDS pandemic continues to increase due to excessive use/ or misuse/abuse of these drugs by HIV/AIDS patients. Furthermore, these leading nosocomial infectious pathogens may become multi-drug resistance (MDR) since there is a high rate of genetic transfer among them. There is the need for proper culture and antimicrobial sensitivity testing before prescription of antibiotics especially for HIV patients. Secondly, potent classes of antibiotics such as the carbapenems and monobactams that are rarely used in many developing countries can be made available as a potential substitute to the quinolones.

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