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Full Length Research Paper

Systematic variations in drug resistance among some enteric gram-negative bacilli isolated from humans and sewage

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This work was designed to undertake a comparative analysis of the drug resistance pattern of enteric bacteria isolated from humans and those isolated from the multifarious microbial environments of the sewage. Human and sewage isolates of enteric Gram-negative bacilli were examined for resistance to ten antibacterial agents. A total of 2400 bacterial isolates (from human n = 1404, and sewage n = 996) isolated over a consecutive three – year (2007 to 2009) period were studied. They include species of *Enterobacter, Escherichia coli, Klebsiella, Proteus, Pseudomonas* and *Salmonella.* Source distribution of resistant isolates showed that sewage isolates were significantly ($p < 0.05$) more resistant than human isolates to most of the drugs tested. High correlations of up to 0.938 between resistance to drugs of sewage and human isolates showed that variation in resistance between the two groups was systematic. Resistance selection and sustenance occur more in the sewage than in human gut but the mechanisms for resistance development are similar, differing only in rate.

Key words: Gram-negative bacilli, human isolates, sewage isolates, drug resistance, resistance correlation.

INTRODUCTION

Enteric Gram-negative bacilli are a heterogeneous group of bacteria made up of members of the family Enterobacteriaceae and others that share a common anatomic location and resemble them but differ, for example in that they are strict aerobes. Most of the members of the former group inhabit the intestinal tracts of man and other animals as normal flora while many members of the latter group are transit inhabitants. For example, while members of the genus *Pseudomonas* can be considered inhabitants of the intestinal flora, only about 10% of humans carry them in the intestinal tract (Lewinson and Jawetz, 2002).

Sewage contains, among other things, human excreta and wastewater originating from a community and the industries there in. It also contains metal ions and other substances that have been implicated in the selection and sustenance of multidrug resistant (MDR) bacteria (Eze et al., 2009; Guardabassi and Dalsgaard, 2002; McArthur and Tuckfield, 2000). As an ecological system, sewage connects antibiotic selective environments such as hospitals, (chemical) industries, farms and slaughter houses to natural environments. In addition, multiple drug resistant bacteria occurring in sewage effluents have sufficient time to transfer resistance genes to indigenous aquatic bacteria if and once they are released into natural (aquatic) environments. This makes sewage important as a reservoir and a possible vehicle for the dissemination of antibiotic resistance (genes) in the indigenous microflora of aquatic and terrestrial environments. The contact between sewage borne bacteria and commensal or pathogenic bacteria occurs through farm animals, vegetables, or when the sewage bacteria enter the atmosphere by means of (water) droplets or (soil) dust. Once in the atmosphere, bacteria can be distributed over large geographical areas and subsequently return to the earth through rain, snow or dry fall (Yuriewa et al., 1997), thus aiding in the distribution of (multidrug resistant) bacteria or their genes.

Considering this melting pot status and vehicular potentials of sewage and the bioactive interactions that take place in it, it was deemed appropriate to undertake a comparative analysis of the antibiotic resistance pattern

of enteric Gram negative bacteria isolated from humans and those from sewage. The aim is to determine which of the two environments harbours more resistant isolates of members of the same genus and also, to determine by statistical (correlation) analysis, whether any variation(s) in resistance between the two groups was random or systematic. This will be of obvious interpretative relevance in the search for the mechanism of development and transfer of resistance among bacteria.

MATERIALS AND METHODS

Isolation and identification of bacteria

Stool and sewage samples were collected over a three year (2007, 2008 and 2009) period. Samples were collected from informed volunteers in Nsukka and Enugu metropolis and University of Nigeria Nsukka sewage treatment plant within the same period in the same chosen months (February, March, July, August, October and November) of each year. Months were chosen to reflect seasonal peaks. Human stool samples were collected from donor adults (18 years and above) using sterile plastic containers. Within 8 h of collection, a suspension of the formed or semi formed stool samples was made in 1 ml of sterile peptone water (MERCK, 7228) according to the method of Cheesbrough (1984). A loopful of each suspension was inoculated on MacConkey agar (Lab M) plates by streaking. Plates were incubated for 24 h at 37°C and colonies were classified as lactose fermenters or non-lactose fermenters based on pigmentation (Levy et al., 1988). Using the five – colony method (Osterblad et al., 1995), five colonies from each plate were chosen by selecting colonies with different colonial morphologies. These were further purified twice, transferred onto nutrient agar (NA) (Lab M) slants, incubated for 24 h at 37°C and stored at 4°C until need.

Isolation of bacteria from sewage followed the method earlier described (Eze et al., 2009). All bacteria isolates (human and sewage) were subjected to standard morphological and biochemical tests. They were subsequently identified based on the criteria of Krieg and Holt (1984) and Cowan and Steel (1965).

Antimicrobial susceptibility tests

Susceptibility test was done by the disc-diffusion method in accordance with CLSI (NCCLS) (2006) and the Swedish Reference group for antibiotics (Olsson–Liljequist et al., 1997). Isolates were screened for susceptibility to a panel of ten antibiotic (Optun Nig.) discs viz: tarivid (10 µg), peflacine (10 µg), ciprofloxacin (10 µg), augumentin (30 µg), gentamicin (10 µg), streptomycin (30 µg), ceporex (10 µg), nalidixic acid (30 µg), septrin (30 µg), and ampicillin (30 µg). Standardized Mueller – Hinton broth cultures of isolates were assayed for sensitivity to these antibiotics on Mueller – Hinton agar plates as earlier described (Eze et al., 2009). Control plates were inoculated and incubated without antibiotic discs. After incubation of test plates (24 h at 37°C) and measurement of inhibition zone diameters, susceptibility ranges were scored following CLSI (NCCLS) (2006), Anon (1988), De La Rosa et al. (1993) and Prescott et al. (1999).

Statistical analysis

Using the statistical package for social sciences (SPSS) (Version 14) Inc (444N Michigan, USA), analysis of variance (ANOVA), Pearson correlation and Post Hoc Tests were carried out to determine any significant associations between antibiotic resistances (outcome variable) and sampling sites (human and sewage). The level of correlation of antibiotic resistance among the enteric Gram negative bacteria from the two sources was also determined. Significance level was scored at 0.05 and 0.01 level (2 – tailed).

RESULTS

Enteric Gram negative bacteria isolated from sewage within the study period are species of the genera *Acinetobacter, Alcaligenes, Enterobacter, Escherichia, Klebsiella, Pseudomonas, Salmonella* and *Proteus.* Organisms that were not isolated from humans in statistically significant numbers (for comparative reasons) were excluded from further analysis. They include member of the genera *Alcaligenes* and *Acetobacter*. Annual distribution of overall resistance showed that percentage resistance increased from 2007 (0.056%) to 2008 (0.092%) and became slightly lower in 2009 (0.091%). Compared with human isolates on the basis of mean resistance, more resistant strains were isolated from sewage (2.61%) than from humans (0.016%). This mean difference is significant at the 0.05 level.

Species of *Enterobacter* isolated in 2007 were more resistant to seven of the 10 drugs than those isolated from humans within the same period. Similarly, sewage isolates (SS) of 2008 showed higher percentage resistance to ciprofloxacin (8.33%), augumentin (25.0%), streptomycin (16.67%), septrin (16.67%) and ampicillin (25.0%) than human isolates (HS) which had corresponding percentage resistance of 2.12, 2.86, 3.24, 5.23 and 5.48%, respectively against the test drugs (Figure 1).

As shown in Figure 2, strains of *Escherichia coli* isolated from sewage in 2009 showed higher resistance to tarivid (22.22%), augumentin (22.22%), gentamicin (11.11%), streptomycin (11.11%), septrin (22.22%) and ampicillin (33.33%) than those isolated in 2007 and 2008 from the same source. *E. coli* strains isolated from humans in 2009 were only more resistant to nalidixic acid (2.34%) and ceporex (1.82%) than sewage isolates of the same year that showed no resistance to both drugs.

Human Isolates of *Klebsiella* spp. of 2007 were more resistant to nalidixic acid (6.00%), ceporex (4.00%) streptomycin (10.00%), gentamicin (2.00%), and peflacine (6.00%) than SS of the same period which showed no resistance to nalidixic acid, ceporex, streptomycin, and gentamicin and 5.56% resistance to peflacine. In contrast SS of *Klebsiella* spp. of 2009 were more resistant than HS of the same period to all test drugs except nalidixic acid (Figure 3).

Percentage resistance of species of *Proteus* isolated from sewage in 2007 was higher than that of human isolates of the same year against augumentin (15.38%) and streptomycin (15.38%). Against these drugs, human isolates had percentage resistance of 4.35 and 13.04%, respectively. HS of *Proteus* (2008) were more resistant than sewage isolates to seven of the ten drugs tested but

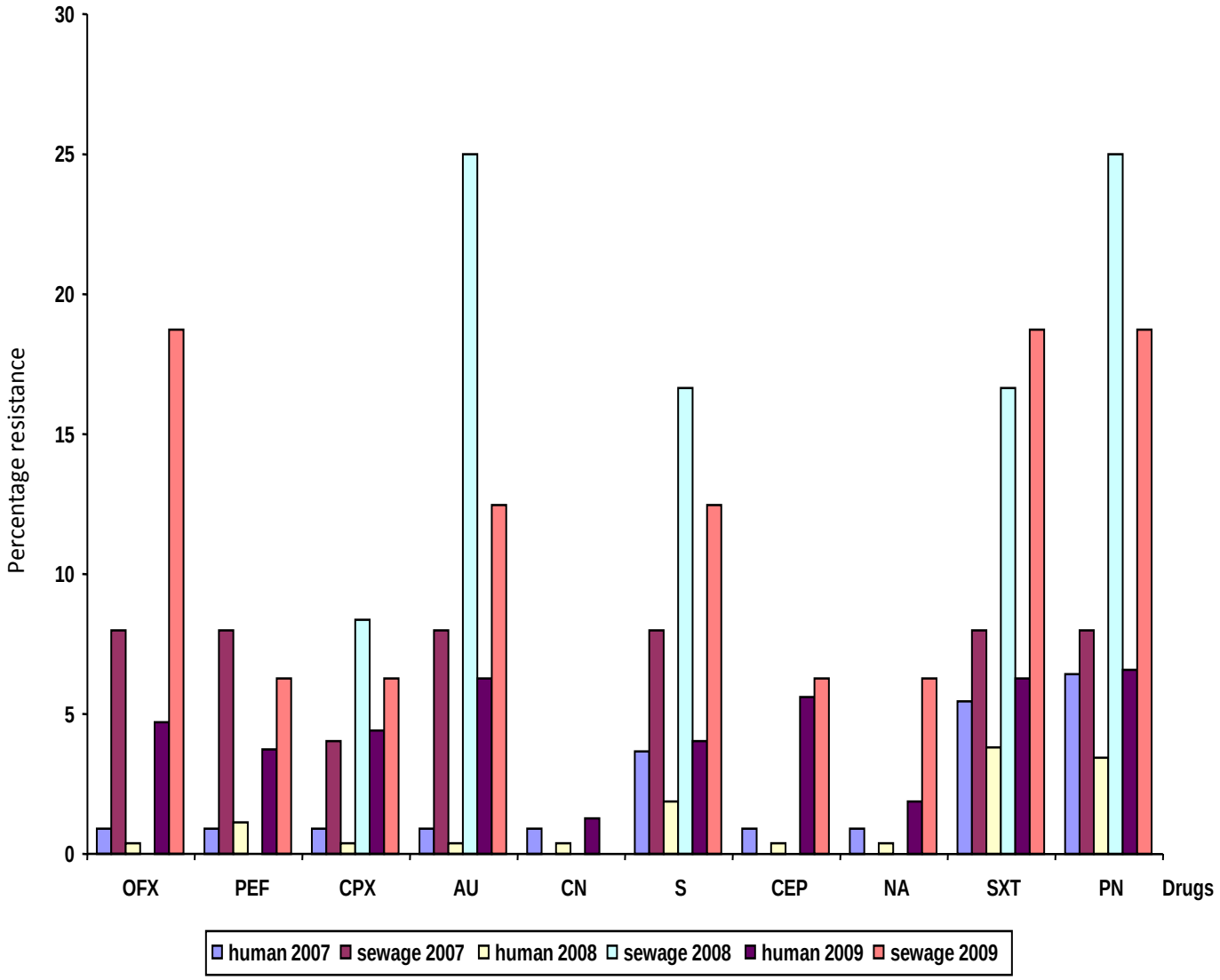

Figure 1. Drug resistance pattern of *Enterobacter* spp. isolated from humans and sewage over a three year (2007, 2008 and 2009) period against commonly available antimicrobial agents. Key: OFX = Tarivid; PEF = Peflacine; CPX = Ciprofloxacin; AU = Augumentin; CN = Gentamicin; S = Streptomycin; CEP = Ceporex; NA = Nalidixic acid; SXT = Septrin, PN = Ampicillin.

2009 sewage isolates were more resistant than human isolates of the same year to all the ten drugs tested (Figure 4). Percentage resistance of *Salmonella* species isolated in 2007 from sewage was higher than HS to six drugs. This contrasts with the resistance pattern of 2008 isolates (Figure 5). The resistance pattern of *Pseudomonas* spp. isolated from both human and sewage in 2007, 2008 and 2009 reveals higher percentage resistance of sewage isolates of 2007 to 2008 of the 10 drugs than human isolates. In contradistinction, human isolates of both 2008 and 2009 were more resistant to at least seven drugs than sewage isolates of these periods (Figure 6).

Pearson correlation analysis showed a 0.904 correlation in the mode of developing multiple drug resistance (MDR) between *E. coli* strains isolated from sewage and *Enterobacter* spp. obtained from the same source (Table 1). This correlation is significant at 0.01 levels (2-tailed). Between *Salmonella* spp. isolated from human and *Proteus* spp. (SS), the correlation is 0.948 and this also is significant at 0.01 levels. In contrast, there is a non-significant low correlation of 0.391 between SS of *Klebsiella* and *Enterobacter* spp., and 0.413 between HS of *Enterobacter* spp. and SS of *Klebsiella* spp.

Also statistical analysis of percentage resistances of both human isolate and sewage isolates against individual antibacterial agents revealed significant ($p < 0.05$) differences among some mean percentage resistance values. The difference between mean percentage resistance to tarivid (36.36%) on one hand and septrin

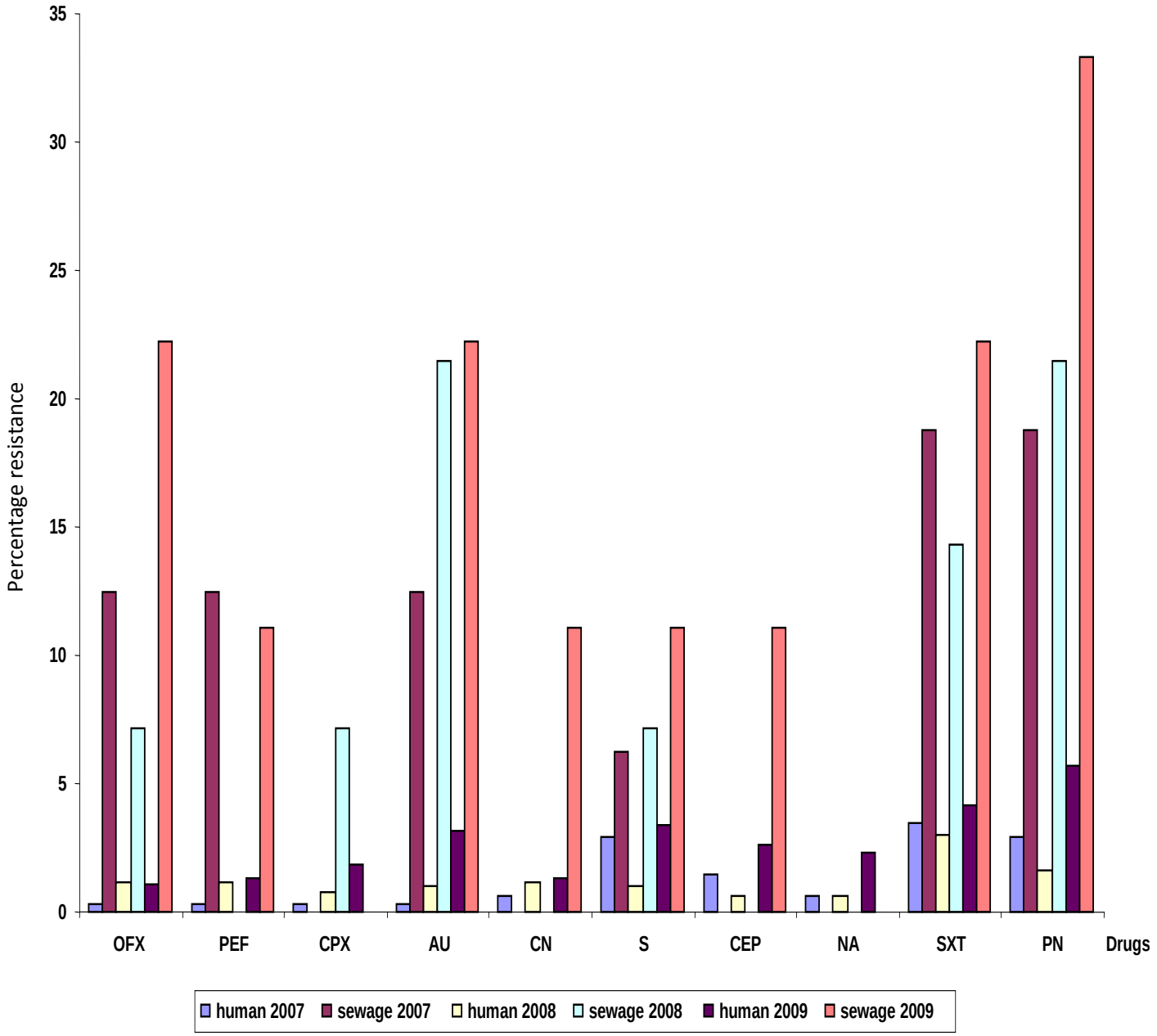

Figure 2. Drug resistance pattern of *Escherichia coli* isolated from human and sewage over a three year (2007, 2008 and 2009) period against commonly available antimicrobial agents. Key: OFX = Tarivid; PEF = Peflacine; CPX = Ciprofloxacin; AU = Augumentin; CN = Gentamicin; S = Streptomycin; CEP = Ceporex; NA = Nalidixic acid; SXT = Septrin, PN = Ampicillin.

(58.69%) and ampicillin (76.60%) on the other hand is significant ($p < 0.05$). The difference between mean resistance to peflacine (32.70) and streptomycin (53.10) is significant at the 0.05 level (Table 2).

DISCUSSION

Human and sewage isolates of enteric gram negative bacilli were examined for resistance to some antibacterial agents and by extension their potentials as reservoirs of multidrug resistance traits. Enteric gram negative bacilli were chosen in this study because of their close association with humans and increasing reports of development of resistance to drug among them. For instance, acquired carbapenemases are known to confer extensive antibiotic resistance to Enterobacteriaceae and represent a public health threat (Struelens et al., 2010, Nordman et al. 2009). Bacteria like *Klebsiella* spp., *Pseudomonas* spp. and *Citrobacter* spp. among others

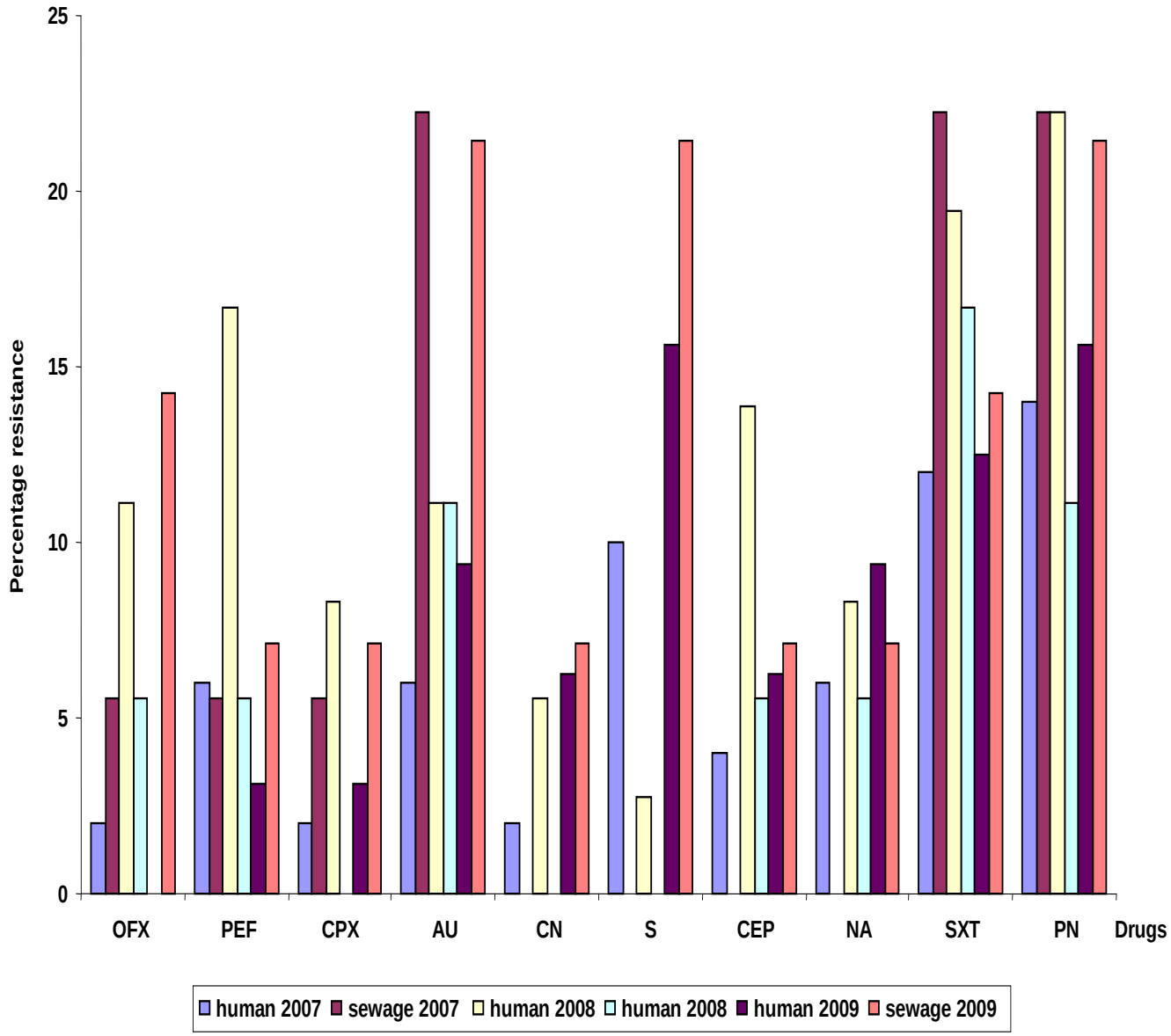

Figure 3. Drug resistance pattern of *Klebsiella* spp. isolated from human and sewage over a three year (2007, 2008 and 2009) period against commonly available antimicrobial agents. Key: OFX = Tarivid; PEF = Peflacine; CPX = Ciprofloxacin; AU = Augumentin; CN = Gentamicin; S = Streptomycin; CEP = Ceporex; NA = Nalidixic acid; SXT = Septrin, PN = Ampicillin.

are generally noted as metallo beta lactamse producers and this confers resistance on these bacteria (Chakraborty et al., 2010). Sewage was chosen because it connects antibiotic selective environments. By this, it is a possible melting pot for exchange of resistance traits among bacteria and is strategic for the dissemination of such traits (Guardabassi and Dalsgaard, 2002; Yuriewa et al., 1997). Within the period of study (2007 to 2009), more resistant bacteria were isolated from sewage in 2008 than in the other two years. Samples were collected at about the same period in the same months of each year. The difference in resistance may therefore be attributable to other variables such as nutrients, suspended solids, rainfall, temperature and pH which were not investigated in this work.

Results of source comparison showed that generally sewage isolates of test bacteria were more resistant to most antibacterial agents than human isolates. Mean percentage resistance values of sewage isolates to all test drugs were higher than those of human isolates (Table 2). This appears to be in tandem with the speculations of Mach and Grimes (1982) to the effect that 2 to 5% of the coliforms (members of the enteric gram negative bacilli group) in sewage contain R plasmids responsible for

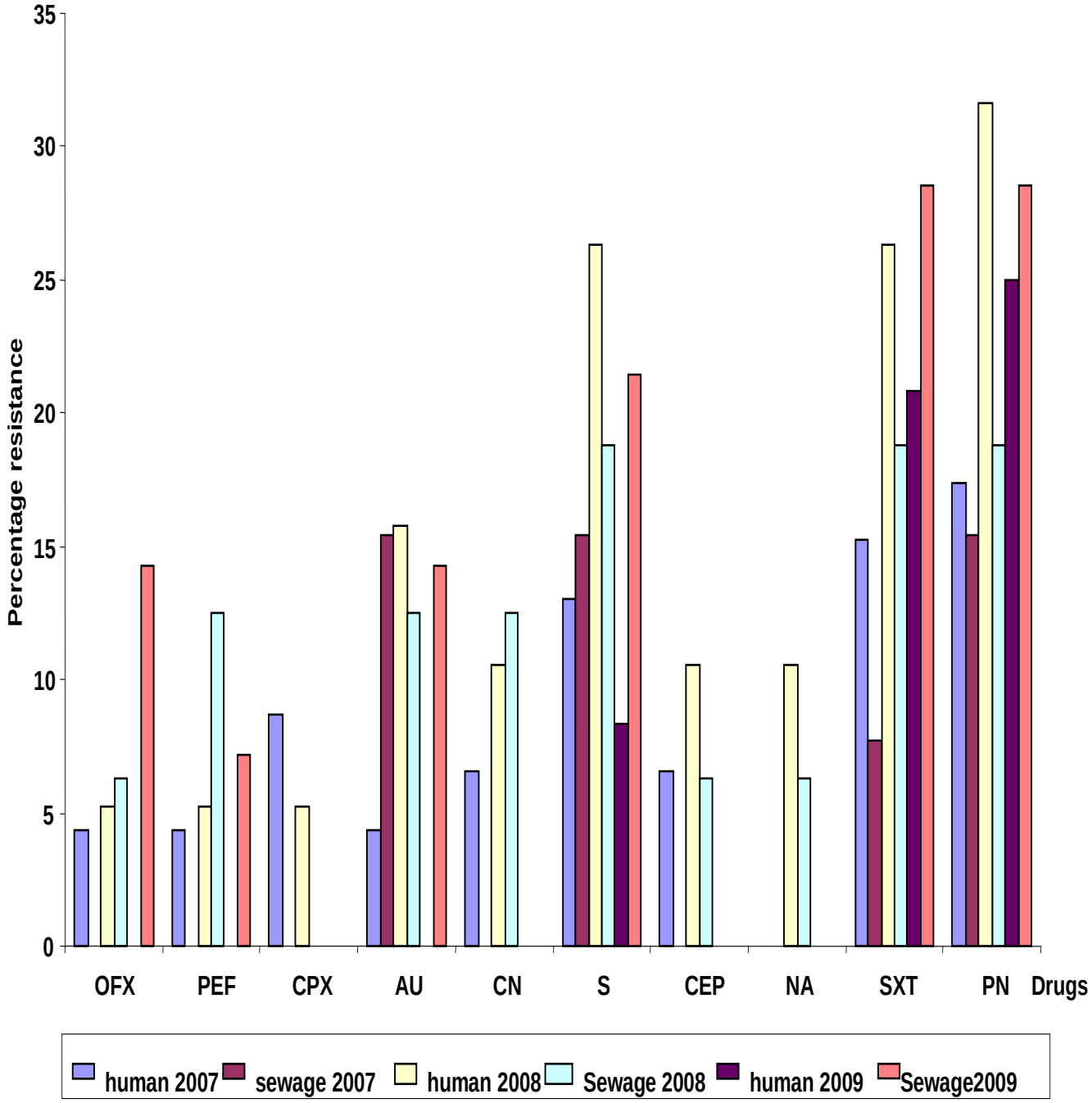

Figure 4. Drug resistance pattern of Protues spp. isolated from human and sewage over a three year (2007, 2008 and 2009) period against commonly available antimicrobial agents. Key: OFX = Tarivid; PEF = Peflacine; CPX = Ciprofloxacin; AU = Augumentin; CN = Gentamicin; S = Streptomycin; CEP = Ceporex; NA = Nalidixic acid; SXT = Septrin, PN = Ampicillin.

drug resistance in these organisms.

The difference in resistance between sewage and human bacterial isolates was analyzed statistically to determine whether it was systematic or random. Being systematic implies that the mechanisms are similar if not the same (Mach and Grimes, 1982). High correlations noticed among some bacteria of different genera isolated from the two sources (Table 1) are indicative of systematic rather than random variation. These results strongly suggest that the antibiotic resistance patterns in these bacterial groups are similar, perhaps showing similar mechanisms for the development of this resistance with a difference only in the rate. Specifically, this points to the presence of a common or closely related

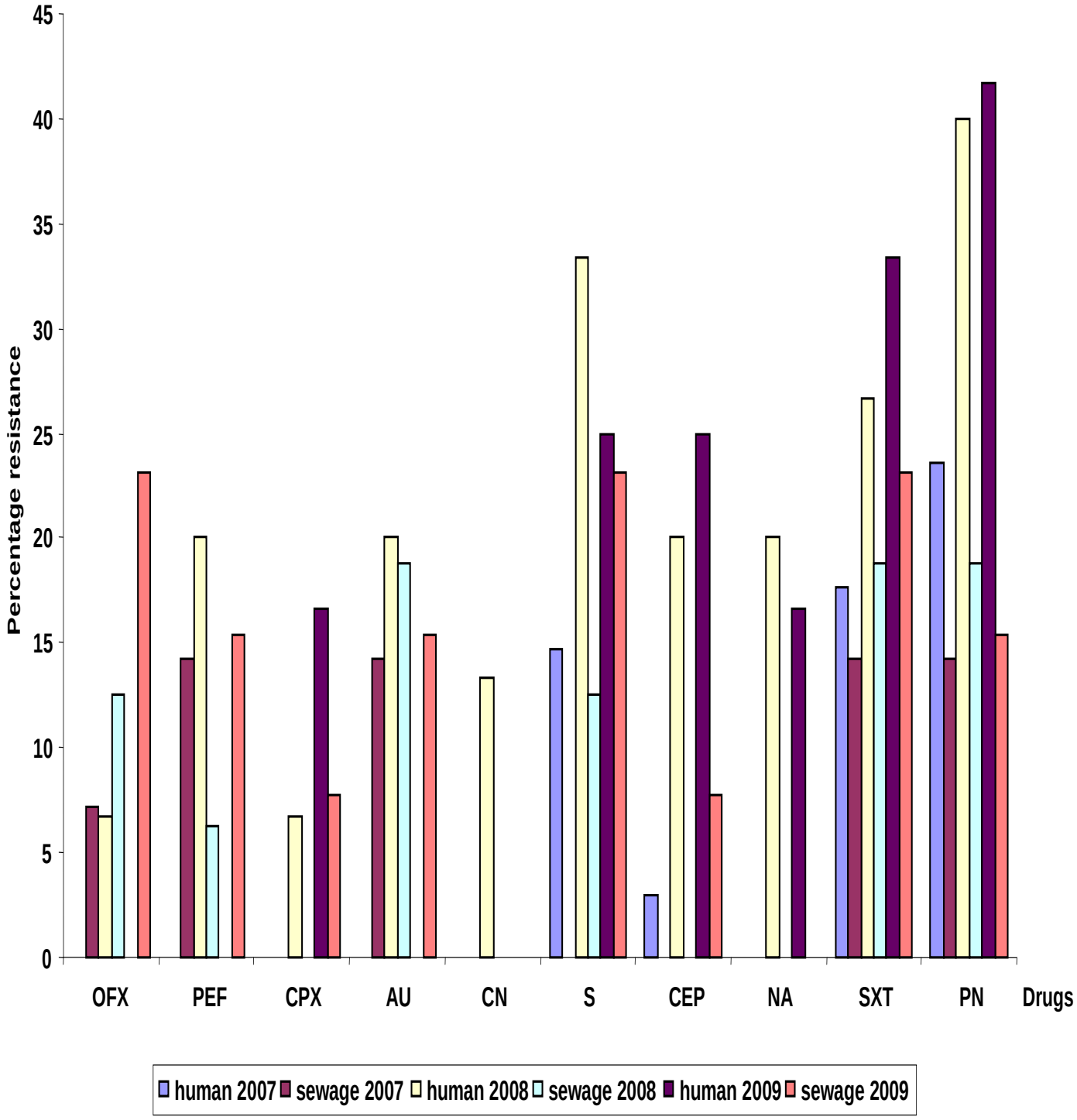

Figure 5. Drug resistance pattern of Salmonella spp. isolated from human and sewage over a three year (2007, 2008 and 2009) period against commonly available antimicrobial agents. Key: OFX = Tarivid; PEF = Peflacine; CPX = Ciprofloxacin; AU = Augumentin; CN = Gentamicin; S = Streptomycin; CEP = Ceporex; NA = Nalidixic acid; SXT = Septrin, PN = Ampicillin.

genetic trait as responsible for resistance among these bacteria. The basis for these observations cannot be fully explained by the experimental demonstration in this work (more analysis is on-going in our laboratories to determine the molecular basis of this). These results are replica and represent an update of earlier unpublished research report (Eze, 2008), confirming the consistency and validity of the systematic nature of the variations in

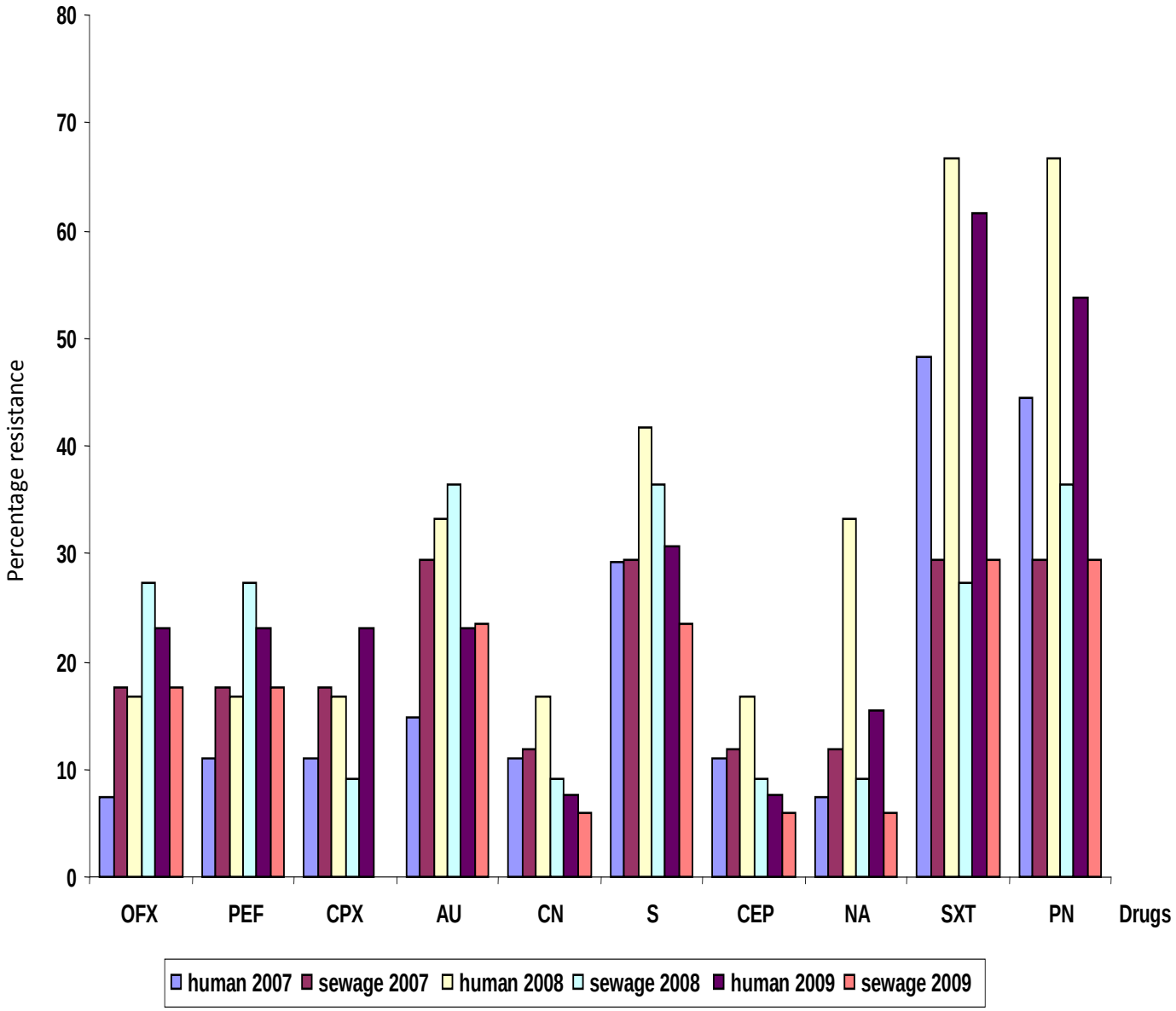

Figure 6. Drug resistance pattern of Pseudomonas spp. isolated from human and sewage over a three year (2007, 2008 and 2009) period against commonly available microbial agents. Key: OFX = Tarivid; PEF = Peflacine; CPX = Ciprofloxacin; AU = Augumentin; CN = Gentamicin; S = Streptomycin; CEP = Ceporex; NA = Nalidixic acid; SXT = Septrin, PN = Ampicillin.

drug resistance observed in this study. What is obvious from this work is that although human excreta is common to both sources of bacterial isolates, resistance selection and transfer occur more in sewage than in the human gut. It is possible that, among other things, factors such as UV radiation and heavy metals present in sewage (Eze et al., 2009; Dhakephalkar and Chopade, 1994; Roane and Kellog, 1996) provide the selective advantage necessary for maintaining multiple resistances among sewage borne bacteria. This has far reaching public health consequences. It is therefore recommended here that in the search for the intractable problem of multidrug resistance among bacteria, attention should be extended to multifarious microbial environments such as sewage.

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Table 1. Correlation matrix of antibiotic resistance among some enteric gram-negative bacteria isolated from humans and sewage (2007 to 2009).

Source	Group	Isolated from sewage						Isolated from human					
		Enterobacter spp.	*Escherichia coli*	*Klebsiellc* spp.	*Proteus* spp.	*Pseudomaonas* spp.	*Salmonella* spp.	*Enterobacter* spp.	*Escherichia coli*	*Klebsiella* spp.	*Proteus* spp.	*Pseudomonas* spp.	*Salmonella* spp.
Sewage	*Enterobacter* spp.	1											
	Escherichia coli	0.904**	1										
	Klebsiella spp.	0.391	0.645*	1									
	Proteus spp.	0.857**	0.831**	0.562	1								
	Pseudomonas spp.	0.938**	0.886**	0.476	0.951**	1							
	Salmonella spp.	0.907**	0.921**	0.446	0.829**	0.940**	1						
Humans	*Enterobacter* spp.	0.799**	0.826**	0.413	0.833**	0.787**	0.771**	1					
	Escherichia coli	0.649*	0.687*	0.491	0.850**	0.709*	0.616	0.927**	1				
	Klebsiella spp.	0.661*	0.722*	0.466	0.817**	0.696*	0.596	0.906**	0.951*	1			
	Proteus spp.	0.681*	0.688*	0.490	0.856**	0.711*	0.594	0.914**	0.964**	0.902**	1		
	Pseudomonas spp.	0.724*	0.748*	0.505	0.868**	0.763*	0.691*	0.929**	0.977**	0.930**	0.955**	1	
	Salmonella spp.	0.882**	0.932**	0.920**	0.954*	0.654*	0.524	0.882**	0.932**	0.920**	0.948**	0.900**	1

**Correlation is significant at the 0.01 level (2 – tailed): *Correlation is significant at the 0.05 level (2 tailed).

Table 2. Mean percentage resistance of bacteria isolated from Human and Sewage against generic antibacterial agents.

Source of isolate	Mean percentage resistance to:									
	OFX	PEF	CPX	AU	CN	S	CEP	NA	SXT	PN
Human (H)	6.33	6.51	6.52	9.64	5.40	16.08	8.69	6.31	23.20	24.70
Sewage (S)	30.03	26.19	7.69	48.63	5.55	37.02	10.62	6.49	35.49	54.90
H + S	36.36	32.70	14.21	58.27	10.95	53.10	19.31	12.80	58.69	79.60

OFX = Tarivid; PEF = Peflacine; CPX = Ciprofloxacin; AU = Augumentin; CN = Gentamicin; S = Streptomycin; CEP = Ceporex; NA = Nalidixic acid; SXT = Septrin; PN = Ampicillin.

REFERENCES

Anon (1988). National Committee for Clinical Laboratory Standards (NCCLS) Supplements M 100-S2. Antimicrob.News Lett., 5: 9-15.

Chakraborty D, Basu S, Das S (2010). A study on infections caused by metallo beta lactmase producing Gram Negative Bacteria in intensive care Unit Patients. Am. J. Infect. Dis., 6(2): 34-39.

Cheesbrough M (1984). Medical Laboratory Manual for Tropical Countries (vol. 11) Microbiology. Butterworth and Co. Ltd Kent. pp. 58-64; 248-273.
CLSI (2006). National Committee for Clinical Laboratory Standards – Performance Standards for Antimicrobial Disk Susceptibility Tests. CLSI 26 (1) Wayne Pa.
Cowan ST, Steel KJ (1965). Manual for the Identification of Medical Bacteria. University Press Cambridge.
Daoud Z, Hobeika E, Choucair A (2008). Isolation of the First Metallo-beta-Lactamase Producing *Klebsiella pneumoniae* in Lebanon. Rev. Esp. Quimioter., 21: 123-126.
De La Rosa MC, Mosso MA, Garcia ML, Plaza C (1993). Resistance to the antimicrobial agents of bacterial isolated from non-sterile pharmaceuticals. J. Appl. Bacteriol., 74: 570 – 577.
Dhakephalkar PK, Chopade BA (1994). High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of *Acinetobacter.* Bio. Metals., 7: 65 – 74.
Eze EA (2008).Human Enteric Gram Negative Bacilli As Resevoirs of Multidrug Resistance Traits. Unpublished Ph.D thesis.University of Nigeria, Nsukka,Nigeria.
Guardabassi L, Dalsgaard A (2002). Occurrence and fate of antibiotic resistant bacteria in sewage. Paper presented to Danish Environmental Protection Agency, 722*:* 1-59.
Krieg NR, Holt JG (1984). Bergey's Manual of Systematic Bacteriology 1: Willian and Wilkins, Baltimore.
Levy SB, Marshal B, Schluederberg S, Rowese D, Davis J (1988). High frequency of antimicrobial resistance in human faecal flora. Antimicrob. Agents. Chemother., 32(12): 1801–1806.
Lewinson W, Jawetz E (2002). *Medical Microbiology and Immunology* (7th ed.). The Mc Graw-Hill Companies Inc Ny. pp. 25-83; 115–137.
Mach PA, Grimes DJ (1982). R-plasmid transfer in a wastewater treatment plant. Appl. Environ. Microbiol., 44: 1395 – 1403.
McArthur JV, Tuckfield RC (2000). Spatial Patterns in Antibiotic Resistance among Stream Bacteria: effects of industrial pollution. J. Appl. Environ. Microbiol., 66(9): 3722–3726.
Nordman P, Cuzon G, Naas T (2009). The real threat of *Klebsiella pneumoniae* carbapenemase producing bacteria. Lancet. Infect. Dis., 9(4): 228-236.
Olsson-Liljequist B, Larsson P, Walder M, Miorner H (1997). Antimicrobial susceptibility testing in Sweden. Methodology for susceptibility testing. Scand. J. Infect. Dis., 105: 13-23.
Osterblad M, Leistvuo T, Huovinen P (1995). Screening for antimicrobial resistance in fecal samples by the replica plating method. J. Clin. Microbiol., 33: 3146–3149.
Prescott LM, Harley JP, Klein DA (1999). Microbiology (4th ed.) McGraw-Hill Companies, N.Y., pp. 213 – 218.
Roane TM, Kellog ST (1996). Characterization of bacterial communities in heavy metal contaminated soils. Cana. J. Microbiol., 42: 593 – 603.
Strelens MJ, Monnet DL, Magiorakos AP, Santos O, Connor F, Gieseckie J (2010). The European NDM-1 Survey Participants. New Delhi Metallo-beta lactamase1- producing *Enterobacteriaceae*: Emergence and response in Europe. Eur. Surveill., 15: 46-19716.
Yuriewa O, Kholodii G, Minakhin L, Gorlenko Z, Kalyaeva E, Mindlin S, Nikiforov V (1997). Intercontinental spread of promiscuous mercury resistance transposons in environmental bacteria. Mol. Microbiol., 24: 321–329.