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Multi-antibiotics Resistant Relatedness of *bla-gene* Encoded Enteric Bacteria harbouring High Molecular R-plasmids.

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ABSTRACT

Increase prevalence of multi-resistant enteric bacteria isolates encoded with high mobile R-plasmid causing enteric infections was examined among the community residents in Abeokuta, Nigeria. Random cluster sampling of 251 fecal samples of community residents were cultured for enteric bacteria and biotyped. Disc diffusion and Micro-broth dilution assay were used to determine antibiotic susceptibility while R-plasmid was profiled with photo-gel documentation. Antibiotic resistance relatedness was detected using DendroUPGMA construction utility software. Of all isolates obtained, 31.3% were *Escherichia coli Klebsiella oxytoca* (19.5%), *Pseudomonas aeruginosa* (15.3%) and *Shigella specie* (2.0%). Significant high rate of 62.6% showed resistant to Cefuroxime, 61.6% to Ampicillin and Augmentin (54.2%) while 44.7%, 38.9% and 33.9% resist Cotrimoxazole, Ciprofloxacin and Tetracycline respectively at MIC ≥16 µg/ml (p= 0.004). Only 54.1% harboured high molecular weight R-plasmid (>11.0kbp) and 2.7% having <5kbp R-plasmid weight. Two distinct clusters revealed significant multi-antibiotic resistant relatedness. Cluster A enteric isolates harboured similar R-plasmid of only one bands with high molecular weight more than 11kbp while Cluster B divided into subgroup *a* and subgroup *b* comprising different enteric species having similar high molecular weights with high antibiotic resistant expressing more than two plasmid bands showing computed cophenetic correlation of 0.94. Cluster analysis reveal a related high level multi-antibiotics resistant enteric bacteria strains among the community residents suggesting a continuous dissemination and imminent outbreak of resistant enteric pathotypes with resultant epidemic proportion.

***Keywords**: Antibiotics, Resistant, R-Plasmid, Bacilli*

1. INTRODUCTION

Resistance to commonly known efficacious antibiotics in recent past has become a global burden, due to its various mechanism of resistance exhibited by many enteric bacteria isolates (Liebert *et al*., 2000). Particular concern is widespread acquisition of other resistant elements while their regional variation in resistance pattern is not unusual (Giske *et al*; 2008). Emergence of enteric resistant pathogens has been linked to increased mortality rates in recent African outbreaks (Iruka *et al*., 2007;

Akinduti *et al*; 20011), characterized with various life-threatening disease condition with a very high epidemic potential. The impact of resistance in mortality and morbidity rate in developing countries is overwhelming in major feature resistance to commonly used antibiotics (Correia *et al*; 2003). These multidrug-resistant strains were probably arose following the acquisition of high molecular resistance plasmids DNA bearing a class 1 integron in many commonly used antimicrobial agents for empiric management of enteric infections (Naik, 2006). Unregulated use of antimicrobial agents in clinical cases has contributed to increased emergence of multi-resistant enteric pathogens to various classes such as beta-lactams, aminoglycosides, fluoroquinolones and more recently, carbapenems (Berglund *et al*., 201).

Moreover, high level mobility of plasmid DNA enhance rapid dissemination of multiple resistant gene among many enteric pathogens causing continuous emergence of related resistance pattern characterized with severe intra- or extra-intestinal infections and high morbidity (Akinduti *et al.,* 2016).

Transfer of conjugative R-plasmids is considered to be the most common mechanism for genetic exchange between bacteria, as plasmid conjugation can occur at high frequency and is capable of co-transfer of several resistance genes within same or different bacterial species showing related multi-resistance activities with high enteric infection burden (Thi *et al*., 2007).

This study was therefore aimed at investigating the relatedness of multi-antibiotic resistance pattern of enteric bacteria harbouring R-plasmid DNA in Abeokuta community in Nigeria.

2. METHODS

Sample collection: Fresh faecal samples of two hundred and fifty one (251) community residents who were not on antibiotics, either as therapy for gastro-intestinal complication or prophylaxis were selected for culturing and isolation of enteric bacteria.

Culture and phenotypic characterisation: All the faecal samples collected were cultured on MacConkey agar and Salmonella-Shigella agar, and incubated at 37^{O}C for 18-24hours and further purified. Colonial and cellular morphology of each enteric bacteria isolates were examined (Cowan and Steel, 2004) while haemolytic activity was detected.

Biotyping: Each bacteria isolate was further characterised using Analytical Profile Index for Enterobacteriaceae (API 20E) tests.

Antimicrobial susceptibility: Using disc diffusion method (Jamil *et al*., 2009), each bacteria strains were assayed for their susceptibilities to the following: Tetracycline (30µg), Cefuroxime (30µg), Augmentin (10/20µg), Ceftazidime (30µg), Gentamycin (10µg), Cotrimoxazole (5/25µg), Ofloxacin (10µg), Ampicillin (10µg) and Ciprofloxacin (10µg). Briefly, pure enteric isolates of 0.5McFarlan turbidity was spread on Mueller-Hinton Agar and and listed antibiotic disc was placed on the inoculated agar and incubated at 37^{0}C for 18 to 24 hours. The inhibition zone of each antibiotic was measured and interpreted according to CLSI guidelines (CLSI, 2014).

Minimum inhibitory concentration determination (MIC): Micro-broth dilution method was used to determine the MIC of the antibiotics such as Tetracycline, Cefuroxime, Augmentin, Ceftazidime, Gentamycin, Cotrimoxazole, Ofloxacin, Ampicillin and Ciprofloxacin (diluted between 0.5 to 64ug/ml) in micro-plate bioassay. MIC of each antibiotic to the isolates was interpreted according to CLSI guidelines (CLSI, 2014). The antibiotic showing MIC $\geq$16ug/mL was selected as resistant enteric isolates.

Minimum bacteriacidal concentration (MBC) assay: Two fold well showing no growth including MIC wells were plated onto Nutrient agar plates and incubated at 37^{0}C for 18 to 24 hours to determine

the MBC while growth of bacteria at ≥ 64µg/ml to more than two classes of antibiotics is termed multi-antibiotic resistant strains.

Characterization of R-plasmid DNA: R-Plasmid DNA of resistant enteric isolates was extracted using alkaline lysis method (Birnboim and Doly, 1979). Complete submerged horizontal agarose gel electrophoresis was employed to determine the size and fragments of the isolated plasmids. Briefly, 20µl of stained plasmid DNA (containing 15µl of plasmid DNA and 5µl of gel green dye) of each extract was electrophoresed using 1% agarose gel concentration in TBE running buffer at 60 mA and 220V for 2 hours. The gel was visualized by UV-transilluminator and photomicrograph to generate size, lane and profile of the plasmids.

Dendrogram analysis of multi-resistant enteric bacteria: Multi-antibiotic resistant relatedness of enteric bacteria strains was detected using DendroUPGMA construction utility program to produce a dendrogram from a set of variables indicated as susceptible and resistant with the distance matrix to calculate a similar matrix and transform similar coefficients into distances making a clustering using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm (Castillo-Vera *et al.,* 2012).

Data analysis: The significance of the resistant bacteria strains obtained was determined using Chi square (χ**2**) and Fisher's exact tests taking p value < 0.05 at confidence interval of 95%. Statistical analysis was performed using SPSS version 20.0.

3. RESULTS

1. Occurrence rate of enteric bacteria isolates: Among 251 fecal samples collected, *Escherichia coli* was the highest (31.3%), followed by *Klebsiella oxytoca* (19.5%), *Pseudomonas aeruginosa* (15.3%), *Enterobacter aerogene* (9.4%), and lowest *Shigella specie* (2.0%) as shown in Figure 1.

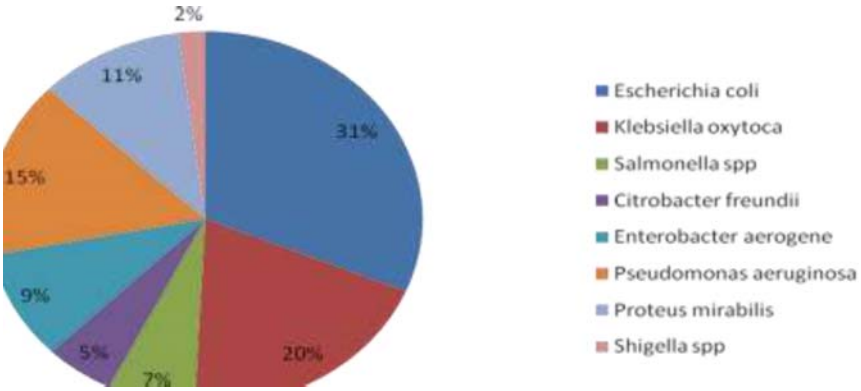

Figure 1; Occurrence rate of common enteric bacteria isolates obtained from community residents

2. Susceptibility testing: Among the enteric bacilli, 62.6% show significant resistant to Cefuroxime (30µg); 61.6% to Ampicillin (10µg) and 54.2% to Augmentin (10/20µg) while lower rate of 15.3% and 19.5% were resistant to Tetracycline (30µg) and Ofloxacin (10µg) respectively (Table 1). Resistance rate of 44.7%, 38.9% and 33.9% was shown to Cotrimoxazole, Ciprofloxacin and Tetracycline respectively with MIC ≥16 µg/ml while less resistance rate of 13.8%, 14.6% and 17.2% to Cefuroxime, Augmentin and Ampicillin were respectively recorded (χ^2 = 33.582; p= 0.004).

Table 1; Susceptibility pattern of enteric bacteria isolates to commonly used antibiotics.

	Antibiotic susceptibility pattern (N=406)			
Antibiotic(µg/mL)	S n(%)	I n(%)	R n(%)	***MIC* *(≥*16* µg*/ml)***
Tetracycline (30)	170(41.9)	174(42.9)	62(15.3)	21(33.9)
Cefuroxime (30)	62(15.3)	90(22.2)	254(62.6)	35(13.8)
Augmentin (10/20)	125(30.8)	61(15.0)	220(54.2)	32(14.6)
Ceftazidime (30)	181(44.6)	110(27.1)	115(28.3)	29(25.2)
Gentamycin (10)	143(35.2)	176(43.3)	87(21.4)	18(20.7)
Cotrimoxazole (5/25)	165(40.6)	147(36.2)	94(23.2)	42(44.7)
Ofloxacin (10)	285(70.2)	102(25.1)	79(19.5)	17(21.5)
Ampicillin (10)	30(7.4)	126(31.0)	250(61.6)	43(17.2)
Ciprofloxacin (10)	188(46.3)	128(31.5)	90(22.2)	35(38.9)

Key: S=Susceptible, I=Intermediate, R= Resistant, **N**= Total number of enteric bacteria isolates obtained; **n**=number of bacteria isolates; **%**=Percentage of bacteria; ***=CLSI (2012)**. (χ^2=31.094; p=0.009).

3. Minimum bactericidal concentration (MBC): Of the resistant enteric isolates shown in Table 2, 14.3%, 13.4% and 9.1% of *Citrobacter freundii*, *Escherichia coli* and *Proteus mirabilis* respectively showed resistance to more than two classes of antibiotics at MBC ≥ 64 µg/ml.

Table 2; Minimum bacteriacidal concentration (MBC) of the commonly used antibiotics against the resistant enteric bacteria isolates.

Bacteria isolates	**MBC ≥ 64ug/ml**		**Antibiotic resistance profile**
	n/N	(%)	
Eschericia coli	17/12	13.4	TRIS, GEN, CPX, CFX, AUG, CFZ, OFX, TET
Klebsiella oxytoca	6/79	7.6	CFX, AMP, CFZ, OFX, COT, GEN
Salmonella spp	1/27	3.7	AMP, GEN, AUG, CFZ, OFX, CPX, TET
Citrobacter freundii	3/21	14.3	AMP, GEN, OFX, COT, TET, CFZ
Enterobacter aerogene	1/38	2.6	TET, CFZ, OFX, COT, GEN, CFX, AUG
Pseudomonas aeruginosa	5/62	8.1	AMP, TET, GEN, CFZ, CPX, COT, CFX, AUG, OFX
Proteus mirabilis	4/44	9.1	AMP, CPX, CFZ, CFX, TET, GEN, AUG
Shigella spp	0/8	0.0	
Total	**38/406**	**9.4**	

Key: **n**=number of multi-resistant bacteria isolates; **N**=Total number of bacteria isolates; **%**=Percentage of multi resistant bacteria; **TET**=Tetracycline; **CFX**=Cefuroxime; **AUG** = Augmentin; **CFZ**=Ceftazidime; **GEN** = Gentamycin; **COT**=Cotrimoxazole; **OFX**=Ofloxacin; **AMP**=Ampicillin; **CPX**=Ciprofloxacin

4. R-Plasmid DNA: In Table 3, highest rate of 54.1% harboured high molecular weight R-plasmid (>11.0kbp) and 2.7% have low molecular weight R-plasmid (<5kbp) while 21.6% possessed no R-plasmid. Figure 2 shows R-plasmid DNA separation on agarose gel.

Table 3; Multi-resistant enteric bacteria R-Plasmid DNA profile

R-Plasmid weight characterizatio	Number of isolates n(%)
None	08(21.6)
Low MW (<0.5kbp)	01(2.7)
Normal MW (0.6-1.0kbp)	09(24.3)
High MW (>1.1kbp)	20(54.1)

Key: N=Number of bacteria isolates profiled for plasmid; n=number of isolates with plasmid, %= percentage of MAREBS with plasmid, MW=Molecular weight, kbp=kilo base pair

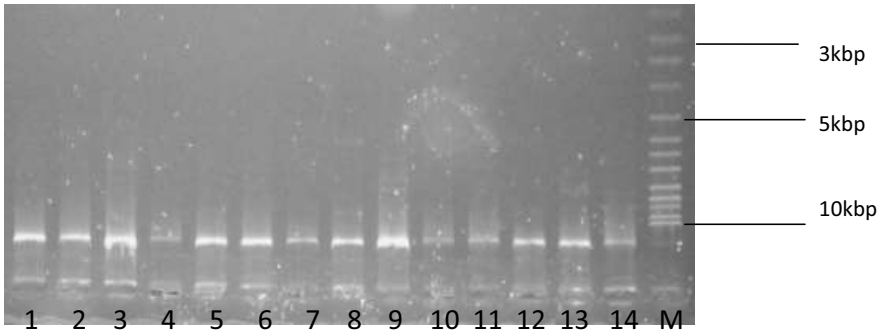

Figure 2; showing DNA bands of R-plasmid MAREBS in Agarose gel.

5. Antibiotic resistant relatedness: Among the multi-resistant enteric bacteria isolate profiled for antibiotic and R-plasmid in Figure 3, two major clades A and B were revealed with significant antibiotic relatedness. Clade A bacteria isolate harboured similar R-plasmid of only one bands with high molecular weight of more than 10kbp while Clade B subgroup *a* and subgroup *b* comprise of different enteric species having similar high molecular weights showing related resistant to more than two classes of antibiotics with some isolates expressing more than two plasmid bands.

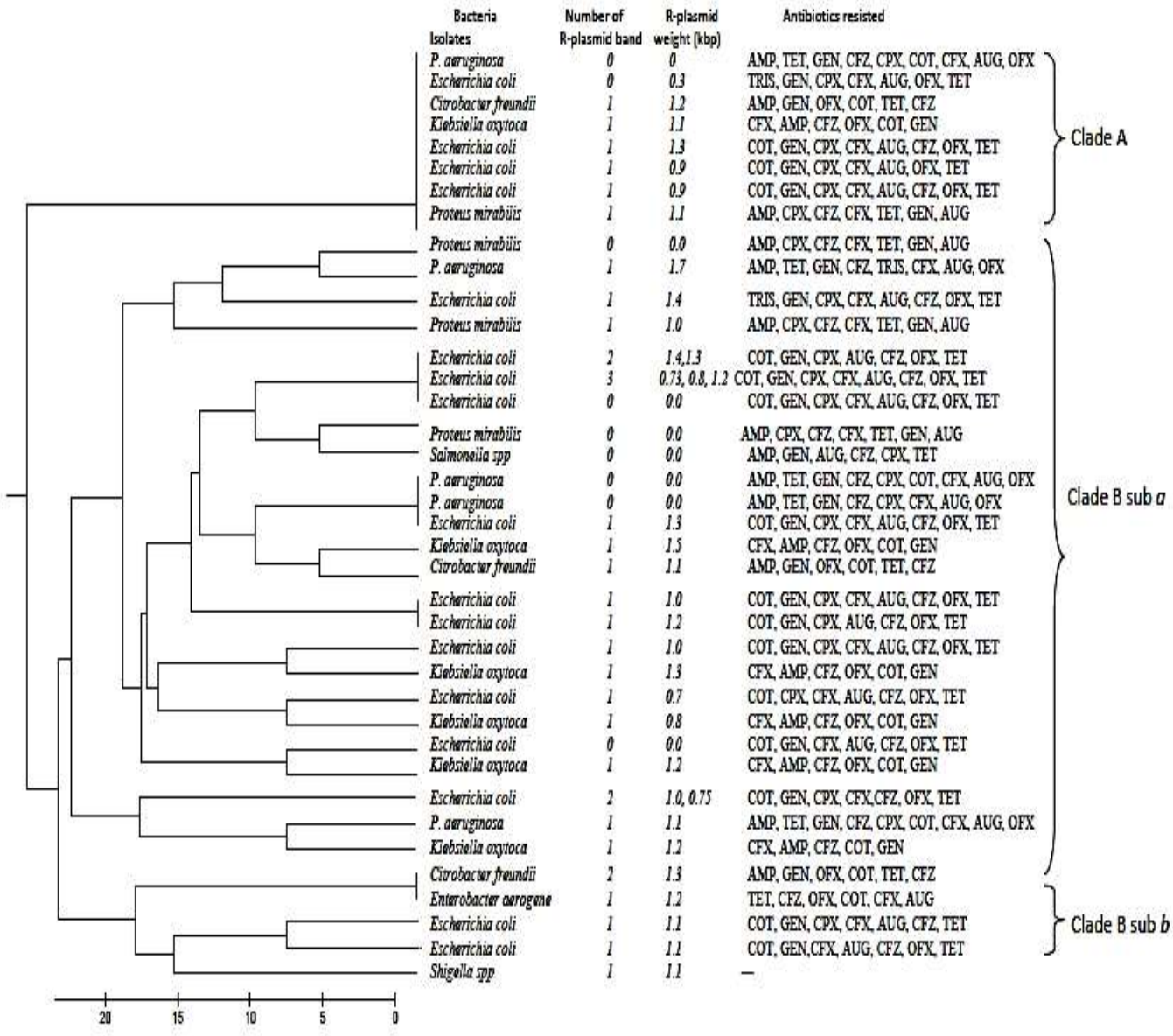

Figure 3: Antibiotic resistant relatedness of enteric bacteria isolates from community residents

4. DISCUSSION

Investigation of emerging strains of multi-resistant enteric bacteria in Abeokuta, Nigeria reveal occurrence rate of *Escherichia coli* (31.3%) *Klebsiella oxytoca* (19.5%) and *Pseudomonas aeruginosa* (15.3%) that were significantly distributed among the subjects suggesting a widespread prevalence of enteric pathotypes which was similarly reported (Ogbu *et al.,* 2008; Ifeanyi *et al.,* 2010). This trend indicate spersistent increase of infectious enteric bacilli among the populace with its attendant severe intestinal diseases that could lead to death mostly among children (Amir *et al*., 2015). The adverse effect of enteric infectious diseases in many poor communities is considerable high usually among the low-income earners who frequently consume unhygenically prepared food and water.

It was amazing to note that most of the enteric bacilli showed significant resistance to commonly used antibiotics. More than 50% of the enteric bacilli showed significant resistant to Cefuroxime, Ampicillin and Augmentin compare to Tetracycline and Ofloxacin which is a reflection of excessive and often unnecessary use of antibiotics in humans and animals (Bonten *et al*., 2001; Qadri *et al.,* 2005). In Abeokuta, the situation is more complex than simple antimicrobial unregulated use but with diverse multiple factors including austere fiscal economic measures, poor health guidelines, incapability of the government agencies to enforce environmental cleanliness and poor health orientation (WHO, 2008). Similar earlier reports by Motayo *et al*., (2013) and Akinduti *et al.,* (2014) further support increasing dissemination of high level multi-resistant enteric bacilli. This gloomy perception could become a threat and a more worrisome situation, particularly in persistent infections leading to high morbidity. Enteric bacteria showing high MIC level of resistance may be maintained by anti-bacterial drug selection and their frequent occurrence in this locality may be linked to over-use of antibacterial agents which needs to be regularized.

In recent past, surveillance of resistant enteric isolates among healthy population has demonstrated that commensal constitute a rich reservoir of genetic material from which pathogens can readily transfer resistance on mobile genetic elements (Mohammadi *et al*., 2011). This observed resistance profile reveals a desperate situation that call for urgent intervention as a result of increasing low cidal activity of commonly used antibiotics that steadily depreciate and could worsening infection control due to the consumption and domestic use of unclean water, poor sanitation, persistent failure to comply with antibiotic use regimen.

R-plasmid is a major identified mechanism of antibiotic resistance transfer, which is a key agent of change in microbial populations (Actis *et al.,* 1999). It has been found to promote rapid dissemination of resistance traits thereby propagating spread of extended resistance to various antibiotics (Akinduti *et al*., 2011). Of multi-resistant enteric bacilli profiled, 54.1% harboured >11kbp R-plasmid and 2.7% having low molecular weight R-plasmid (<5kbp) that was similarly reported (Adagbada *et al.,* 2014;Akingbade *et al.,* 2014) in South west Nigeria. High molecular weight R-plasmids harboured by these resistant bacilli showed a prolific rate of resistance transfer posing a dangerous threat and imminent outbreak. Plasmid-mediated resistance to several potent antibiotics has become a huge challenge to effective prevention of many enteric infections, thereby imposing huge costs of treatment on the society (de Kraker *et al.,* 2011; Finley *et al*., 2013).

Not only has plasmid–mediated resistant being a monster to this community, it has further revealed a pending epidemic incidence and spread of community-acquired resistant enteric bacilli with related resistant pattern. Two major classes of resistant enteric bacilli belonging to

clade A and B were revealed to be circulated in this community with significant antibiotic resistance relatedness. Clade A bacteria isolates harboured similar R-plasmid of only one band with high molecular weight of more than 10kbp.
Related R-plasmid with similar pattern of antibiotics resistance demonstrates a wide distribution of similar resistance profiles in diverse bacilli with high molecular weight plasmid. Diversity in resistance pattern shown by Clade B subgroup *a* and subgroup *b* comprising different enteric species expressing similar high molecular weights R-plasmid with related resistance is indication of newly emerging strains (Selim *et al.,* 2012). This high level of diversity in the plasmid expression among Clade B bacilli gives impression of rapid acquisition of resistant plasmid between two or more genus of the enteric bacteria with implication that plasmids with identical resistance profiles are generally closely related with high tendency for plasmid gene recombination (Thomas *et al*., 2005). Evidence of R-plasmid would enhance flow of genetic information between novel plasmid pairs (Furuya *et al.,* 2005; Camargo *et al.,* 2005). Therefore, broad-scale inter-plasmid gene transfer, probably involving a range of mechanisms, including recombination, transposition and integration could further enhance high level dissemination of resistant enteric bacilli in this community (Carattoli *et al*., 2001).

Conclusion: Plasmid–mediated resistance is highly prevalent revealing a Cluster distribution of high level multi-antibiotics resistant relatedness among the enteric bacteria strains found in this community. This indicates a continuous dissemination and imminent outbreak of community-acquire resistant enteric pathotypes with high potential of transfer.

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