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Molecular typing of clinical and non-clinical *Escherichia coli* from Rivers State, Nigeria using amplification-based RAPD and ERIC-PCR typing methods

Dibia C.[1] and Otokunefor K.[1*]

[1]Department of Microbiology, Faculty of Science, University of Port Harcourt, PMB 5323 Port Harcourt, Nigeria.

Abstract

Due to the diverse nature of *Escherichia coli*, typing of these organisms is essential. This can provide key epidemiological information not just on the strains in circulation and their relationship, but also on the development and spread of drug resistant clones. Affordable PCR-based typing techniques have resulted in an increase in typing studies particularly in resource limited settings. But information from Nigeria on strain typing is limited. This study was therefore aimed at typing clinical and non-clinical strains of *E. coli* from Nigeria using the PCR based Random Amplification of Polymorphic DNA (RAPD) and Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) typing methods.

***E. coli* was isolated from clinical and non-clinical sources using Eosin Methylene Blue agar. Characteristic green metallic sheen colonies associated with *E. coli* were purified, identities determined using standard biochemical tests and confirmed using molecular methods. Susceptibility testing was carried out using the modified Kirby-Bauer disc diffusion test and RAPD and ERIC-PCR typing carried out on 48 confirmed *E. coli* isolates as previously described. ERIC-PCR typing resulted in the generation of 38 unique patterns with a 0.94 diversity. Nine of these were singletons comprised of only a single isolate, while the remaining 29 patterns were grouped to 10 clusters. RAPD gave similar results of 37 patterns, 9 singletons, 0.94 diversity and 13 clusters. For ERIC-PCR, all isolates with identical patterns were from the same source. RAPD, however, had identical patterns present in isolates from different sources.**

Using the ERIC-PCR typing method, this study was able to identify non-clinical isolates as distinct from the clinical *E. coli* isolates as evidenced by 100% identical ERIC-PCR profiles of isolates from the same source and a lack of 100% relatedness in isolates from different sources.

Keywords: *E. coli*, genetic diversity, ERIC-PCR, RAPD, Nigeria
[*]**Corresponding author:** +234 8051844470 kome.otokunefor@uniport.edu.ng

Introduction

Escherichia coli is well known as one of the most commonly isolated bacteria in the clinical microbiology laboratory. Together with *Staphylococcus aureus*, this species of organism is responsible for most cases of multidrug resistance. Despite this, *E. coli* is not an obligate pathogen but also occurs as a commensal found in various environments (Proencal et al., 2017) It is often sometimes necessary to carry out typing studies in order to determine relatedness of strains of a particular bacterial type. This would provide key epidemiological information on presence or absence of clonal spread, strain distribution and possible data on development and spread of drug resistance, especially when comparing organisms from different environments (Ruppitsch 2016).

A number of typing strategies exist which differ in reproducibility, sensitivity, specificity and cost (Li et al., 2009). PCR-based typing methods involving a single primer pair are increasing in their popularity due to their comparable sensitivity and specificity and much lower costs and complexity than gold standard methods such as multilocus sequence typing (MLST) and pulse field gel

electrophoresis (PFGE) (Li et al., 2009). Two key PCR-based methods are the Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) typing method and the Random Amplification of Polymorphic DNA (RAPD) method. These methods have been widely used in typing various strains of *E. coli* globally but particularly in resource limited countries (Dhanashree and Mallya 2012; Nielsen et al., 2014; Pusparini et al., 2018; Movahedi et al., 2021; Tanko et al., 2021), but information on the use of these methods in typing of *E. coli* strains from Rivers State, Nigeria is lacking.

A recent study (Otokunefor et al., 2020) showed that ERIC-PCR could be used for typing of members of the Enterobacteriaceae in Port Harcourt, Rivers State, but the sample size was limited and the study did not focus on *E. coli* specifically. This study was therefore aimed at typing clinical and non-clinical strains of *E. coli* isolated from Rivers State, Nigeria using two PCR-based methods in order to assess the relatedness of isolates which could point at spread from one environment to another.

Methods

Test Isolates

Escherichia coli were isolated from clinical and non-clinical sources (poultry, soil, urine and stool) using the Eosin Methylene Blue (EMB) agar. Characteristic green metallic sheen colonies associated with *E. coli* were purified and identities determined using standard biochemical tests (Cheesebrough 2006; Cowan and Steel 1985). The identities of these isolates were further confirmed using the EC16 primers (F 5'-GACCTCGGTTAGTTCACAGA-3' and R 5'-CACACGCTGACGCTGACCA-3') which amplify *E. coli* specific 16s RNA gene fragments (Islam *et al.,* 2016) from DNA extracted using the Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech, Ltd., Taiwan), according to manufacturer's recommendation.

Ethical Consideration

Ethical approval was obtained from the ethical committee of the University of Port Harcourt Teaching Hospital (UPTH) where the clinical samples were obtained (UPTH/ADM/90/S.11/VOL.XI/1110).

Antibiotic Susceptibility Profile typing

Antibiotic susceptibility was then carried out on the isolates using the Kirby-Bauer disc diffusion test (Bauer et al., 1966). In brief, a bacterial suspension corresponding to 0.5 MacFarland standard was inoculated on a Mueller Hinton Agar plate using a sterile swab stick. Following a 15 min pre-incubation at room temperature, the antibiotic multidisc was applied to the surface of the agar plate and the whole set up incubated for 24 hours at 37°C. Zones of inhibition (mm) were then determined and data interpreted using the CLSI standard (CLSI 2016).

Following this, a binary code for each isolate was then developed as adapted from a previous publication (De Arruda et al., 2003) whereby presence of resistance was noted as "1" and absence of resistance as "0".

Molecular typing

Molecular typing of 48 confirmed *E. coli* isolates was carried out using the ERIC-PCR and RAPD typing methods as previously described (Xia et al., 2012; Marialouis and Santhanam 2016; Otokunefor et al., 2020). This involved amplification with ER1-5'ATGTAAGCTCCTGGGGATTCAC3' and ER2-5'AAGTAAGTGACTGGGGTCAGCG3' for the ERIC-PCR and 5'AACGCGCAAC3' for the RAPD typing. Similar amplification conditions were used for both. For ERIC-PCR, amplification conditions involved: 3 mins at 95°C, 40 cycles of denaturation at 95°C for 3 minutes, annealing at 54°C for 1 minute, extension at 72°C for 3 minutes and the final extension at 72°C for 7 minutes. RAPD differed with an annealing at 37°C for 1 minute

Following separation and visualization on a 2% agarose gel, a binary code was created for each isolate using a scoring template of 1 for the presence of a band and 0 for the absence of a band (De Arruda et al., 2003), with each band position serving as a locus.

Based on the binary data, dendrograms were developed using Jaccard's similarity coefficient data by the unweighted pair-group method using arithmetic means (UPGMA), using the DendroUPGMA server (http://genomes.urv.cat/UPGMA) (Garcia-Vallve et al., 1999).

Discriminatory Index

Discriminatory indices were determined for all three typing methods based on Simpson's Index of Diversity as previously described (Hunter and Gaston 1988).

Results

ERIC-PCR generated bands ranging in size from about 100 bp to 3000 bp with a total of 38 binary codes observed for all isolates. Products ranging in size from about 270 bp to 400 bp were more

commonly occurring, with approximately 350 bp fragment observed as the most frequently occurring band (Figure 1). At a genetic distance of 0.6, an analysis of the dendrogram showed 10 clusters comprising more than 1 isolate (Figure 2) and 9 (18.8%) singletons (U01, F17, U29, F03, F29, P20, F06, F43, S10) indicating isolates showing unique profiles.

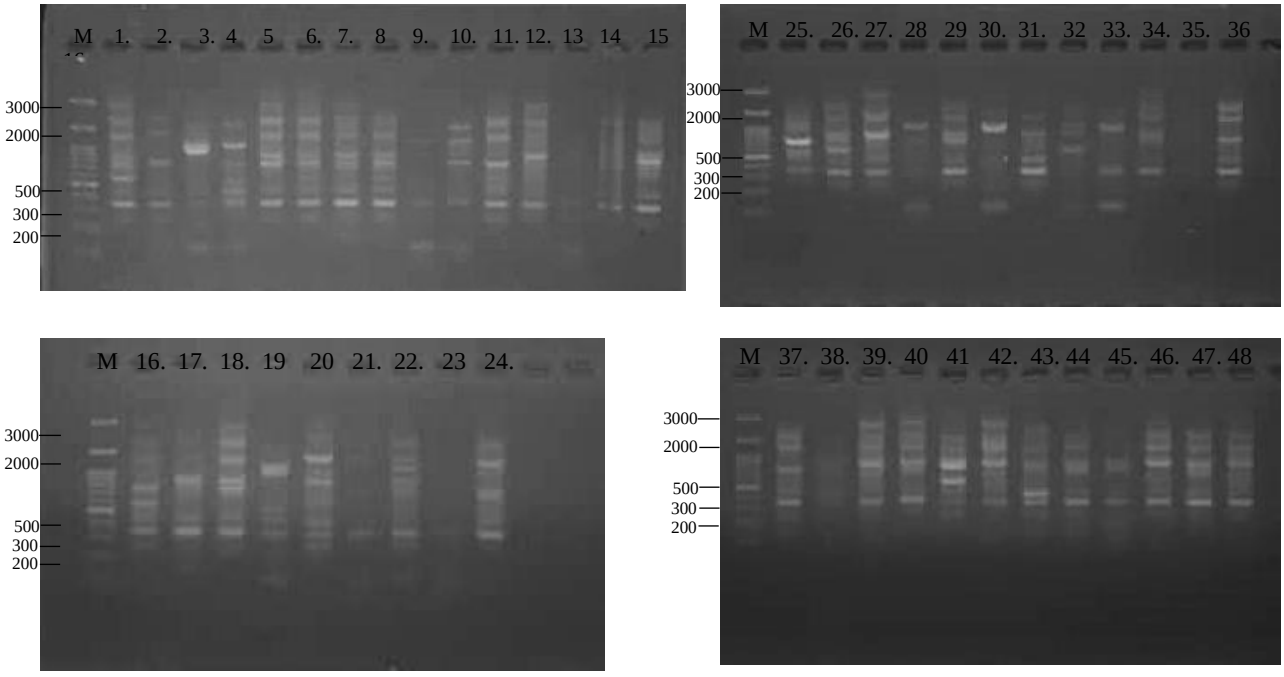


Fig 1: ERIC-PCR profile of 48 *Escherichia coli* isolates run on a 2% agarose gel. Lane M denotes 1 kb DNA ladder (fragment sizes in basepair). Gel shows amplification products ranging in size from 100 bp to 3000 bp in general resulting in unique amplification profiles for each isolate.

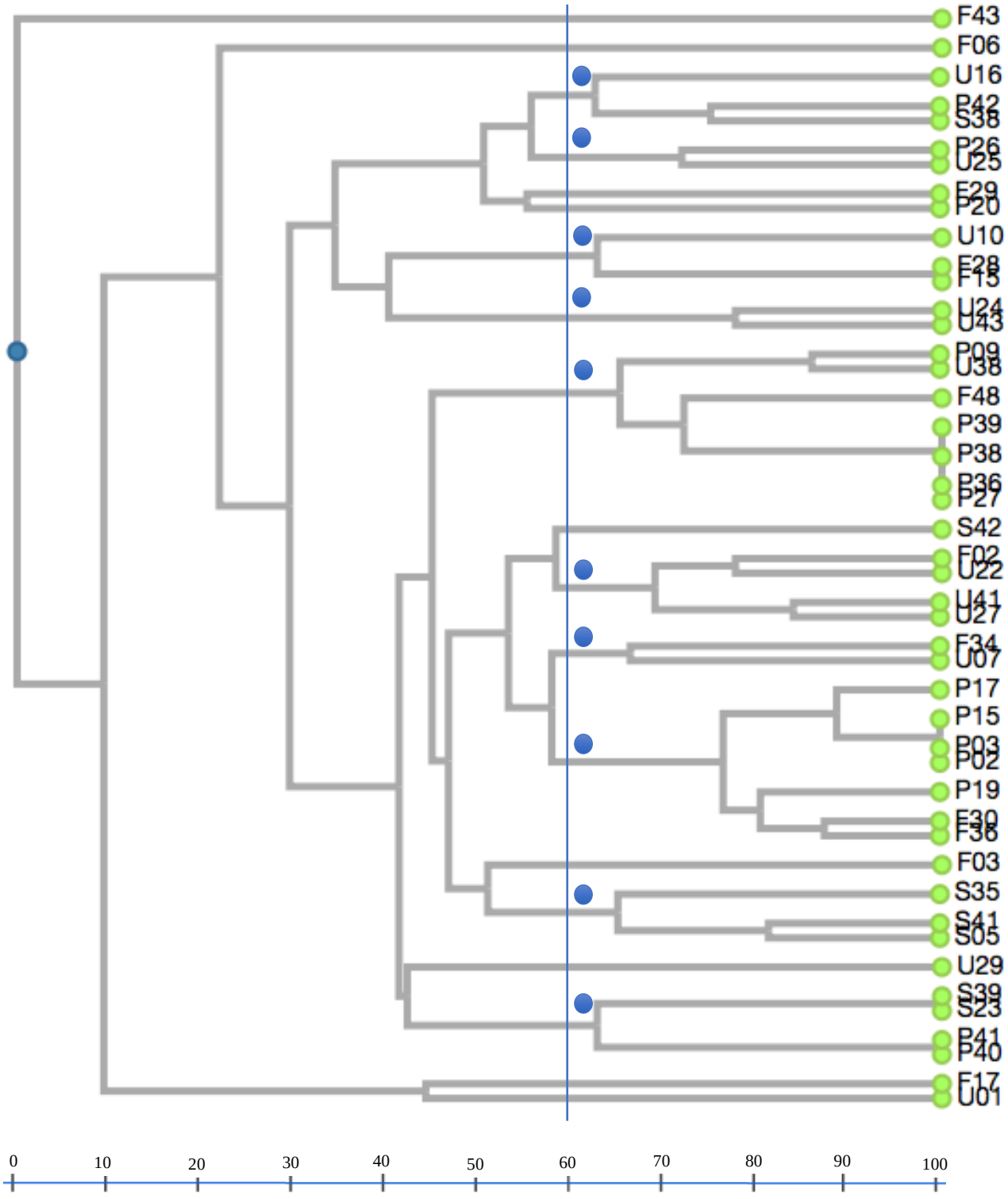


Fig 2: Dendrogram generated by UPGMA clustering of ERIC-PCR profiles of 48 *Escherichia coli* isolates, showing 10 clusters at a 0.6 genetic distance cutoff and identical profiles indicated by more than one isolate at a single node.
Alphabets indicate isolate source: U for Urine, S for Soil, F for Faeces, P for Poultry

The clusters were of varying sizes made up of 2 to 7 isolates (Table 1) with 6 groups of isolates with identical profiles found within 5 of the clusters. Using this method, a greater diversity appears to

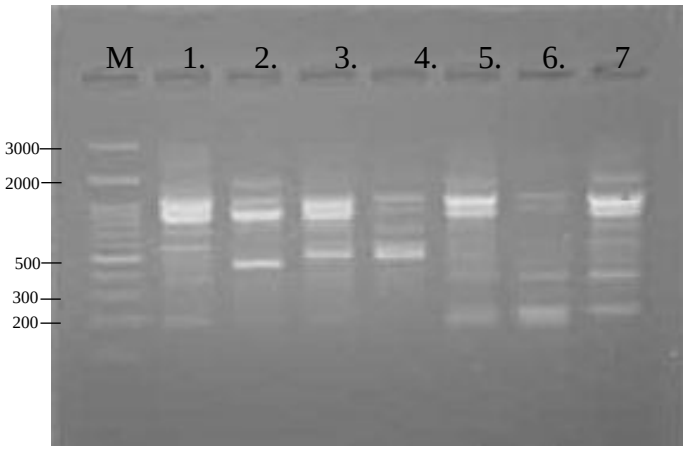
occur with the faecal isolates whereby 41.7% of these (5/12), occurred as singletons. This is in contrast to poultry isolates where a higher level of relatedness was noted with 3 sets of 2, 3 and 4 found to be identical to each other (60%, 9/15). Urine isolates were unique in that no two of them were found to be 100% identical using ERIC-PCR.

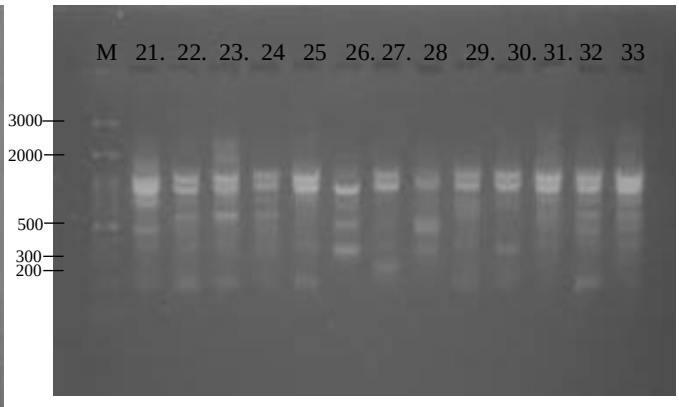
Table 1: Cluster analysis and genetic diversity of the isolates using ERIC-PCR at 60% similarity coefficient cutoff

Cluster ID	Dendrogram Similarity	Frequency of Isolates (%)	Isolate ID	Source			
				Urine	Poultry	Faecal	Soil
1.	63%	3 (6.3)	U16, P42, S38	1	1		1
2.	72%	2 (4.2)	P26, U25	1	1		
3.	63%	3 (6.3)	U10, **F28**, **F15**	1		2	
4.	79%	2 (4.2)	U24, U43	2			
5.	65%	7 (14.6)	P09, U38, F48, **P39**, **P38**, **P36**, **P27**	1	5	1	
6.	71%	4 (8.3)	F02, U22, U41, U27	3		1	
7.	64%	2 (4.2)	F34, U07	1		1	
8.	78%	7 (14.6)	P17, **P15**, **P03**, **P02**, P19, F30, F36		5	2	
9.	65%	3 (6.3)	S35, S41, S05				3
10.	63%	4 (8.3)	**S39**, **S23**, **P41**, **P40**		2		2
Singletons		9 (18.8)	U01, F17, U29, F03, F29, P20, F06, F43, S10	2	1	5	1

Identical isolates are indicated either in bold or bold and underlined where one or two different sets of identical profiles are noted within a cluster respectively.

Typing using RAPD gave very similar results with products ranging from 200 bp to just below 3000 bp (Figure 3), 37 binary codes observed (Figure 4) and 13 clusters of more than 1 isolate and 9 singletons (P03, P20, F17, P09, U43, F29, F15, S23, U29). Larger cluster sizes were however noted here with the highest cluster size containing 10 isolates (Table 2), spanning all 4 sources which did not occur with ERIC-PCR typing. With RAPD, 6 identical isolates were observed in 5 of the clusters. Despite the slight differences, using Simpson's Diversity Index, both methods gave a 0.94 value.





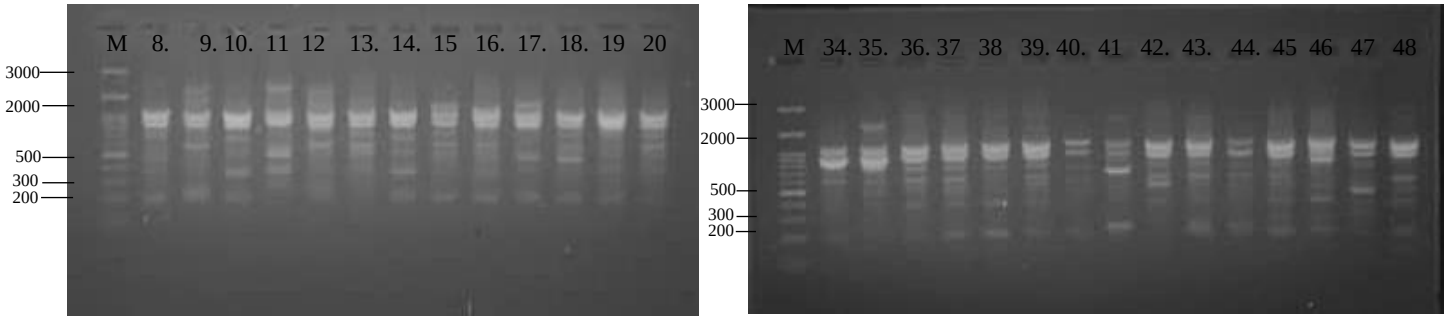


Fig 3: RAPD profile of 48 *Escherichia coli* isolates run on a 2% agarose gel. Lane M denotes 1 kb DNA ladders (fragment sizes in basepair). Gel shows amplification products ranging in size from 100 bp to 3000 bp in general resulting in unique amplification profiles for each isolate.

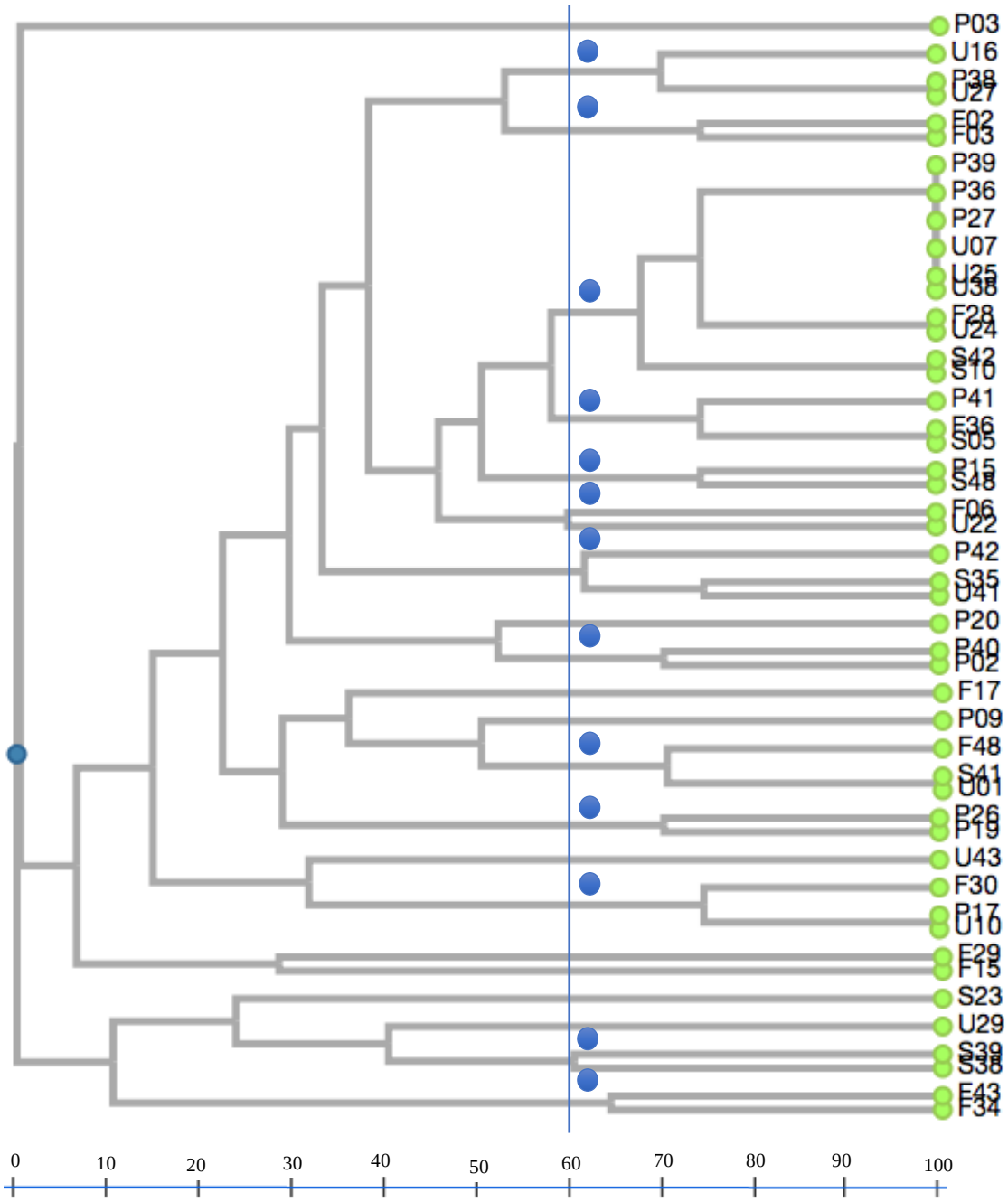


Fig 4: Dendrogram generated by UPGMA clustering of RAPD profiles of 48 *Escherichia coli* isolates, showing 13 clusters at a 0.6 genetic distance cutoff and identical profiles indicated by more than one isolate at a single node. Alphabets indicate isolate source: U for Urine, S for Soil, F for Faeces, P for Poultry

Table 2: Cluster analysis and genetic diversity of the isolates using RAPD typing at 60% similarity coefficient cutoff

Cluster ID	Dendrogram Similarity	Frequency of Isolates (%)	Isolate ID	Source			
				Urine	Poultry	Faecal	Soil
1.	70%	3 (6.3)	U16, **P38**, **U27**	2	1		
2.	75%	2 (4.2)	F02, F03			2	
3.	63%	10 (20.8)	**P39, P36, P27, U07, U25, U38**, F28, U24, **S42**, **S10**	4	3	1	2
4.	75%	3 (6.3)	P41, **F36**, **S05**		1	1	1
5.	75%	2 (4.2)	P15, S48		1		1
6.	59%	2 (4.2)	F06, U22	1		1	
7.	61%	3 (6.3)	P42, S35, U41	1	1		1
8.	70%	2 (4.2)	P40, P02		2		
9.	70%	3 (6.3)	F48, **S41**, **U01**	1		1	1
10.	70%	2 (4.2)	P26, P19		2		
11.	75%	3 (6.3)	F30, **P17**, **U10**	1	1	1	
12.	60%	2 (4.2)	S39, S38				2
13.	65%	2 (4.2)	F43, F34			2	
Singletons		9 (18.6)	P03, P20, F17, P09, U43, F29, F15, S23, U29,	2	3	3	1

Identical isolates are indicated either in bold or bold and underlined where one or two different sets of identical profiles are noted within a cluster respectively.

A total of 24 antibiograms were observed from the 48 isolates (Table 3). 25% of these were unique to single isolates. The antibiogram showing the largest distribution was noted in 10 different isolates encompassing all the sources of isolates. Dendrogram analysis revealed that even more isolates with identical profiles (10) occurring within 5 clusters were observed with antibiogram typing (Figure 5), with no source based relationship (Table 4). This reflected with the 0.90 Simpson's genetic diversity index observed from this method.

Table 3: Antibiogram of all *E. coli* isolates

S/No	Antibiogram (Frequency)
1.	AUG (1)
2.	AUG-TET (4)
3.	CAZ-AUG-TET (2)
4.	CRX-AUG-TET (10)
5.	CXM-AUG-TET (2)
6.	CAZ-CRX-AUG-TET (2)
7.	CAZ-CRX-CXM-TET (1)
8.	CAZ-GEN-AUG-TET (1)
9.	CRX-GEN-AUG CPR (1)
10.	CRX-GEN-AUG-TET (2)
11.	CXM-OFL-AUG-TET (1)
12.	OFL-AUG-CPR-TET (1)
13.	CAZ-CRX-CXM-AUG-TET (3)
14.	CAZ-CRX-GEN-AUG-TET (2)
15.	CRX-CXM-OFL-AUG-TET (1)
16.	CRX-GEN-AUG-CPR-TET (1)
17.	CRX-GEN-CXM-AUG-CPR (1)
18.	CRX-GEN-OFL-AUG-TET (3)

19.	CRX-OFL-AUG-CPR-TET (2)
20.	GEN-OFL-AUG-CPR-TET (2)
21.	CAZ-CRX-GEB-OFL-AUG-TET (1)
22.	CRX-GEN-OFL-AUG-CPR-TET (2)
23.	CAZ-CRX-CXM-OFL-AUG-CPR-TET (1)
24.	CAZ-CRX-GEN-CXM-OFL-AUG-CPR-TET (1)

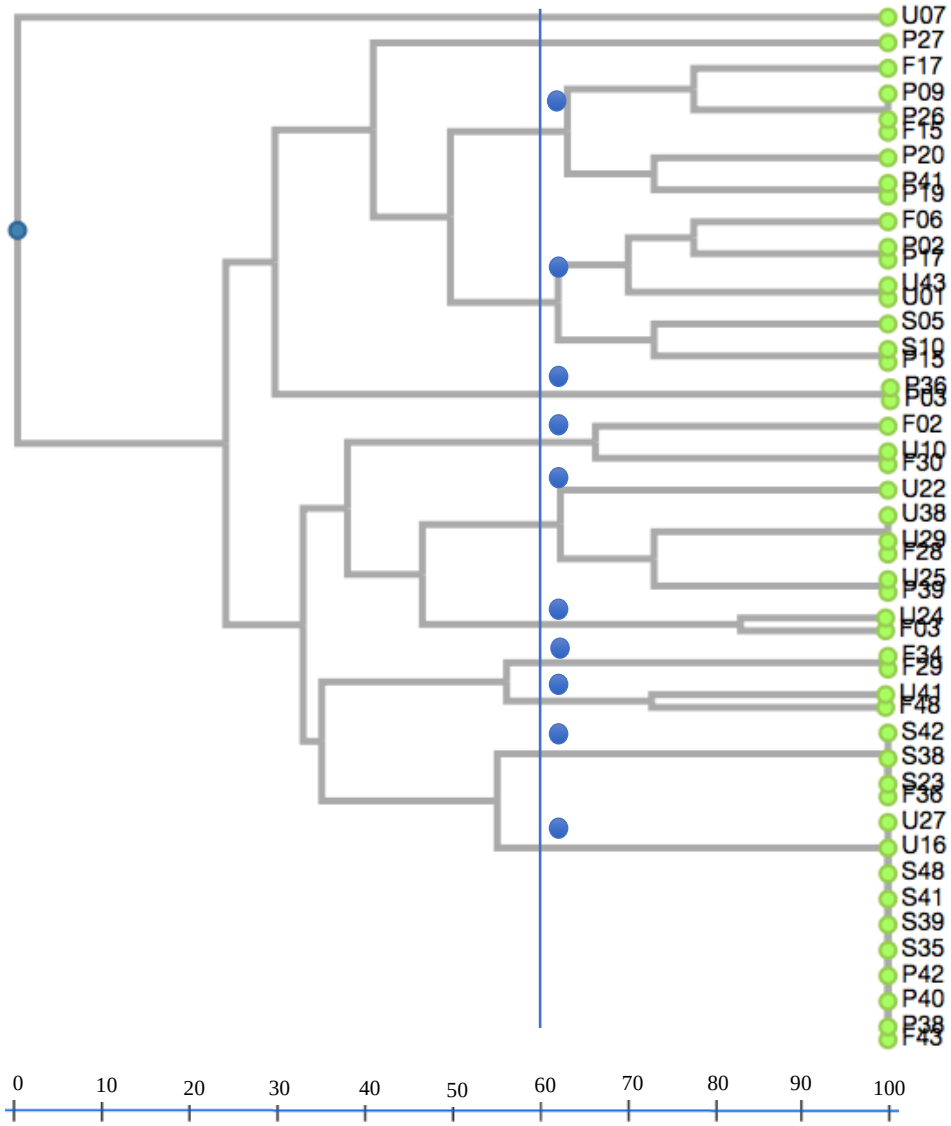


Fig 5: Dendrogram generated by UPGMA clustering of antibiogram profiles of 48 *Escherichia coli* isolates, showing 10 clusters at a 0.6 genetic distance cutoff and identical profiles indicated by more than one isolate at a single node. Alphabets indicate isolate source: U for Urine, S for Soil, F for Faeces, P for Poultry

Table 4: Cluster analysis and genetic diversity of isolates using antibiogram typing

Cluster ID	Dendrogram Similarity	Frequency of Isolates (%)	Isolate ID	Source			
				Urine	Poultry	Faecal	Soil
1.	63%	7 (14.6)	F17, **P09**, **P26**, **F15**, P20, **P41**, **P19**		5	2	

2.	62%	8 (16.7)	F06, **P02**, **P17**, (**U43)**, (**U01)**, S05, **S10**, **P15**	2	3	1	2
3.	100%	2 (4.2)	**P36**, **P03**		2		
4.	65%	3 (6.3)	F02, **U10**, **F30**	1		2	
5.	62%	6 (12.5)	U22, **U38**, **U29**, **F28**, **U25**, **P39**	4	1	1	
6.	83%	2 (4.2)	U24, F03	1		1	
7.	100%	2 (4.2)	**F34**, **F29**			2	
8.	73%	2 (4.2)	U41, F48	1		1	
9.	100%	4 (8.3)	**S42, S48, S38, S23, F36**				5
10.	100%	10 (20.8)	**U27, U16, S48, S41, S39, S35, P42, P40, P38, F43**	2	3	1	4
Singletons		2	U07, P27,				

Identical isolates are indicated either in bold or bold and underlined or bold and bracketed where one, two or three different sets of identical isolates are noted within a cluster respectively.

Discussion

Escherichia coli is notorious as the most commonly encountered pathogen in the clinical microbiology laboratory (Otokunefor and Nyema, 2019). It is also renowned for its ubiquity and antimicrobial resistance. With the current One Health approach recommended particularly as a means of understanding the development and spread of antimicrobial resistance, this study set out to study genetic relatedness of *E. coli* isolates from different sources using ERIC-PCR and RAPD.

ERIC-PCR amplification of *E. coli* specifically has been shown to turn up a range of band sizes with reports of 170 to about 4000 bp, 232 to 2690 bp, 380 to 3280 bp and 300 bp to 4500 bp (Soltani et al., 2012; Ranjbar et al., 2017; Ramakrishna et al., 2022). Though these studies differed in their source of isolates, the variations did not appear to be species based. The bands reported in this present study were within a similar range (100 bp to 3000 bp) as previous reports.

Initial results of this study noting 38 unique binary codes for the 48 isolates (Figure 2) points at a high level of genetic diversity within the isolates, rather than the clonal spread of predominant strains. This was slightly similar to 120 patterns observed from 120 isolates in a 2017 study (Ranjbar et al., 2017). But in contrast to some other studies pointing at predominant strains with high homogeneity between isolates such as 2013 study by Ranjbar and colleagues who reported 175 ERIC-PCR genotypes from 555 isolates (Ranjbar et al., 2013) and a more recent study focusing specifically on uropathogenic *E. coli* (UPEC) which noted just 27 patterns for the 92 test isolates and 13 of these patterns were associated with only a single isolate (Movahedi et al., 2021).

This diversity noted by the number of ERIC-PCR patterns was further confirmed by the 0.90 Simpson's diversity index which ranges from 0 to 1 with 1 signifying a high level of diversity. This falls within the recommended desirable index (≥ 0.90) necessary for interpretation of a molecular typing method with confidence (Pitonda-Silva 2013).

While it is essential for a typing method to differentiate between different strains, for a typing method to be useful, it also needs to be able to class similar isolates into the same group, to determine relatedness (Sabat et al., 2013). Dendrogram analysis of the ERIC-PCR data (Figure 2) revealed that the 29 non-unique profiles could be categorized into just 11 clusters. One of the largest of these clusters comprised of 7 isolates which differed at 3 of the 13 loci only. Of the 6 sets of identical isolates observed (Table 1), all identical isolates were obtained from the same source indicating some level of genetic homogeneity within these sources. Though ERIC-PCR observed related isolates from different sources, all identical isolates were from the same source.

The results of the ERIC-PCR were much different from that of the RAPD when comparing the cluster composition. One significant difference was that following RAPD typing, isolates from different sources gave identical RAPD patterns. This is probably a function of the variation in targets of both typing methods. Unlike ERIC-PCR which targets specific parts of the genome (ERIC sequences), RAPD is not targeted against a specific

genetic locus but rather amplifies random segments of genomic DNA.

Typing of the isolates point at a clonal spread particularly in the poultry environment. Significantly results of this study indicate a lack of delineation between clinical and non-clinical isolates perhaps pointing at spread between the various environments. This highlights the key role intervention studies focused on multiple environments play to reduce the development and spread of antibiotic resistance. Generally isolates from the soil which was chosen for being an environment with minimal human intervention and perhaps strictly commensal isolates, were identified as distinct from the clinical *E. coli* isolates.

Conclusion

Using the ERIC-PCR typing method, this study was able to identify non-clinical isolates as distinct from the clinical *E. coli* isolates as evidenced by 100% identical ERIC-PCR profiles of isolates from the same source and a lack of 100% relatedness in isolates from different sources.

List of Abbreviations

RAPD: Random Amplification of Polymorphic DNA
ERIC: Enterobacterial Repetitive Intergenic Sequences
PCR: Polymerase Chain Reaction
DNA: Deoxyribonucleic acid
MLST: Multilocus sequence typing
PFGE: Pulse Field Gel Electrophoresis

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the ethical committee of the University of Port Harcourt Teaching Hospital (UPTH) where the clinical samples were obtained (UPTH/ADM/90/S.11/VOL.XI/1110).

Consent for Publication

Not Applicable

Availability of Data and Material

The datasets used and/or analysed during the current study are included in this published article.

Competing interests

The authors declare that there are no financial or non-financial competing interests.

Funding

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Authors' contributions: KO conceptualized the work. CD carried out most of the phenotypic analysis under the supervision of KO. KO and CD carried out the molecular analysis. All authors took part in writing the manuscript, read and approved the final manuscript.

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Not Applicable

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