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EXTENDED PHYLOGENY AND PATHOGENECITY OF *E. COLI* IN SCAVENGING LOCAL CHICKEN IN MOROGORO MUNICIPALITY

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Abstract: **The existence of genetic substructure in *E. coli* was revealed by Whittam and colleagues (1983) and was later confirmed by Chaudhuri and Henderson (2012). This research is the first to investigate the various phylo-groups that exist among amongst APEC strains from scavenging local chicken in Morogoro region of Tanzania based on the modern quadruplex method. All eight phylo-groups were detected among the APEC strains in varying percentages. Among the APEC isolates, 63.2%, 68.4%, 78.9%, and 73.7% were found to belong to phylogroups D and B2, C and F respectively, whiles isolates in groups A, B1 and E were 15.8%,10.5% and 5.3% respectively. There was a strong simultaneous occurrence of several iron acquisition genes with of structural gene for microcin *iss thus (iss/iuCD)*, outer membrane protein(*ompA*) thus (ompA/sitEp). Iron acquisition genes and serum resistance genes recorded significant (P<0.05) differences between APEC and non-APEC strains and can thereby be used as markers for APEC isolates identification. The toxin and invasion genes, *astA* and *ibeA* which are normally recorded in minimal percentages were found to be significant determinants of APEC in the study. Funding: This work was supported by the intra ACP mobility scheme**

Keywords: **APEC, scavenging local chicken, revised phylo-grouping.**

1. INTRODUCTION

Escherichia coli are members of the natural microbiota of domestique animals, including chicken (Tenaillon *et al.*, 2010). Although, these bacteria are usually harmless, but part of their population can become extraintestinal pathogenic *E. coli* (ExPEC). ExPEC have a fecal origin and occur asymptomatically in the intestinal tract. They can also colonize extraintestinal niches and cause serious diseases (Starcˇicˇ *et al.*, 2015). Pathogenic strains of *E. coli* have been divided into intestinal (diarrheagenic; DEC) and extra-intestinal (ExPEC) pathogenic E. coli. Avian pathogenic *E. coli* (APEC), a subdivision of ExPEC that produces a systemic disease in poultry and also could serve as a potential zoonotic hazard to humans (Dziva and Stevens, 2008). APEC does not only colonize the intestinal tract of the chicken but also has the ability to disseminate systemically either through intestinal or respiratory mucosa (Leitner and Heller, 1992). The implementation of PCR methods to screen and identify the common virulence genes and phylogroups between different isolates is very critical (Moriel *et al.*, 2010). Virulence genes (VGs) which causes pathogenicity are usually encoded on pathogenicity islands (PAIs), plasmids, and other mobile genetic elements, and hence can be transmitted via horizontal gene transfer (HGT) between various *E. coli* strains (Köhler and Dobrindt 2011).

The population structure of *E. coli* is mainly clonal and strains can be classified into eight phylogenetic groups: A, B1, B2, C, D, E, F, and cryptic clade I (Clemont *et al*., 2013). Factors responsible for the phylogenetic structure of *E. coli* include: environment, gut anatomy, physiology, and diet. In animals, a critical force influencing the phylogenetic structure of the *E. coli* is the domestication status of the host (Escobar-Páramo *et al*., 2006). Domesticated animals have a lower proportion of B2 and A strains than their wild counterparts (Gordon and Cowling 2003).

2. METHODS AND METHODOLOGY

Sample collection and DNA extraction

The study area chosen for this research is the Morogoro municipality. The percentage of the populace that is engaged in livestock keeping and subsistence farming is 33% (Region and District Project Volume XII, 2011). A total of 400 swabs were collected from six different households within the Morogoro municipality. Specifically, oral-pharyngeal and cloacae swabs were collected from each of 200 scavenging local chicken. Half of these birds were kept semi-intensively whiles the remaining were on free-range. The swabs were collected and kept in transport media and transferred into the laboratory on ice

Isolation of *E. coli* and DNA extraction

Procedures used were as described in the Bacterial Analytical Manual (BAM 2007). The organisms were grown on MacConkey and Blood Agar media (OXOID, Hampshire, England) for the isolation.The following biochemical tests were performed to confirm the suspected isolates: Indole, Methyl Red, Voges-Paskeur, Citrate, Triple Sugar Iron and Motility test.

The positive *Escherichia coli* genomic DNAs were extracted by boiling. With modifications was necessary, rapid DNA extraction was done as protocol by Zhang *et al.* (2004).

Polymerase chain reaction

Virulence factor profiling to detect APEC strains

The positive *E. coli* strains, 192 in number, were investigated for various virulence genes by multiplex PCR, with protocol based on Ewers *et al.* (2007). The procedures were performed in 25µlreaction mixture. This includes: 12.5 µl of Taq polymerase (Dream Tag PCR Master mix, InqabaBiotec East Africa Ltd), 0.5 µl of each 100Mm dNTP, 0.1µl (100pmol) oligonucleotide primer pair, 6.9 µl of nuclease-free water and 4µl of template DNA. Primer concentration is 0.4 M. Conditions of the reaction mixtures include: 5mins at 95ºC initial denaturation,94ºC of denaturation for 30s, annealing at 56ºC for 30s, elongation at72ºC for 3minutes at 25 cycles, a final elongation at 72ºC for 10 minutes and a hold at 4ºC. List of primers used is shown in the appendix

Phylo-group profiling

All *E. coli* strains, 192 in number, were screened for the presence of the genes in a quadriplex pcr reaction. The original triplex PCR method by Clemont *et al*., (2000) had three primers: chuA, yjaA and TspE4.C2. This method was later modified by adding arpA targeted primer to act as an internal control for DNA quality and also to distinquish strains belonging to phylo-group F which was earlier misclassified as D. The protocol based on Clemont *et al.* (2013). The procedures were performed in 20µlreaction mixture. This includes: 10 µl of Taq polymerase (Dream Tag PCR Master mix, Inqaba Biotec East Africa Ltd),, 0.4µl (100pmol) oligonucleotide primer pair, 5.9 µl of nuclease-free water and 3µl of template DNA. Primer concentration is 0.4 M. Conditions of the reaction mixtures include: 4mins at 94ºC initial denaturation,94ºC of denaturation for 30s, annealing at 57ºC for 20s, elongation at72ºC for 3minutes at 30 cycles, a final elongation at 72ºC for 5 minutes and a hold at 4ºC. List of primers used is shown in table 1. The isolates whose genotype combinations give 2 possible phylotypes are run on pcr using specific primers. Thus the allele specific for phylo-group E and C are ArpAgpE.f/r and TrpAgpC.f/r respectively. The primer for internal control of E and C specific reaction is trypBA.f/r

The quadruplex genotypes combinations required for assigning phylo-groups are recorded in table 2.

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Table 1: Primer sequences and sizes of PCR products used in the extended quadruplex phylo-typing method.

PCR reaction	Primer ID	Target	Primer sequences	PCR prdt(bp)
Quadruplex	chuA.1b	chuA	5′-ATGGTACCGGACGAACCAAC-3′	288
	chuA.2		5′-TGCCGCCAGTACCAAAGACA-3′	
	yjaA.1b	yjaA	5′-CAAACGTGAAGTGTCAGGAG-3′	211
	yjaA.2b		5′-AATGCGTTCCTCAACCTGTG-3′	
	TspE4C2.1b	TspE4.C2	5′-CACTATTCGTAAGGTCATCC-3′	15
	TspE4C2.2b		5′-AGTTTATCGCTGCGGGTCGC-3′	
	AceK.f	arpA	5′-AACGCTATTCGCCAGCTTGC-3′	400
	ArpA1.r		5′-TCTCCCCATACCGTACGCTA-3′	
Group E	ArpAgpE.f	arpA	5 ′-GATTCCATCTTGTCAAAATATGCC-3′	301
	ArpAgpE.r		5′-GAAAAGAAAAAGAATTCCCAAGAG-3′	
Group C	trpAgpC.1	trpA	5′-AGTTTTATGCCCAGTGCGAG-3′	219
	trpAgpC.		5′-TCTGCGCCGGTCACGCCC-3′	
Internal control	trpBA.f	trpA	5′-CGGCGATAAAGACATCTTCAC-3′	489
	trpBA.r		5′-GCAACGCGGCCTGGCGGAAG-3′	

Table 2: Quadruplex genotypes required for assigning phylo-groups Quadruplex genotype

Quadruplex genotype				Phylogroup
ArpA (400bp)	*ChuA (288bp)*	*YjaA(211bp)*	*TspE4.C2(152bp)*	
+	-	-	-	A
+	-	-	+	B1
-	+	-	-	F
-	+	+	-	B2
-	+	+	+	B2
-	+	-	+	B2
+	-	+	-	A or C
+	+	-	-	D or E
+	+	-	+	D or E
+	+	+	-	E or Clade 1
+	-	+	-	Unknown
-	-	-	-	Unknown

+ Presence of the target amplicon; - Absence of the target amplicon. The isolates whose genotype combinations give 2 possible phylotypes are run on pcr using specific primers

Statistical Analysis

Statistical analysis was done by use of Statistical Parkage for Social Sciences (SPSS). The presence of virulence genes among *E.coli* isolates were categorized as 1 = yes and 0 = no. Pearson's correlation was used to access the frequency of occurrence of the various virulence genes. This frequency was rate from 0 to 1. The range of 1 to 0.1 was considered that highest rate of occurrence and given a color code of red, 0.099 to 0.001 was considered to be mild and given a pale pink

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color code. The least occurrence rate was <0.001 and was assigned a white color. Proportions of various characteristics were tested by use of the chi-square test (x^2). The threshold for statistical significance was indicated in the table with a $P < 0.05$ reflected statistical significance

3. RESULTS

Prevalence of virulence genes

After primary isolation of samples using Macconkey and blood agar media; 192, out of 400 samples were positive for *Escherichia coli.* The suspected positive *Escherichia coli* isolates were all confirmed in the biochemical tests. All suspected isolates, 192, were confirmed to be positive for *E. coli.* Virulence factor profiling revealed *that* all 192 *E.coli* isolates harbored at least of the 16 virulence genes. Also, 19 (9.8%) of them harbored at least 4 of the virulence genes and assigned as APEC isolates (Dziva and Stevens, 2008).

The remaining isolates, 173(90.2%), each containing less than 4 virulence genes were regarded as non-APEC.

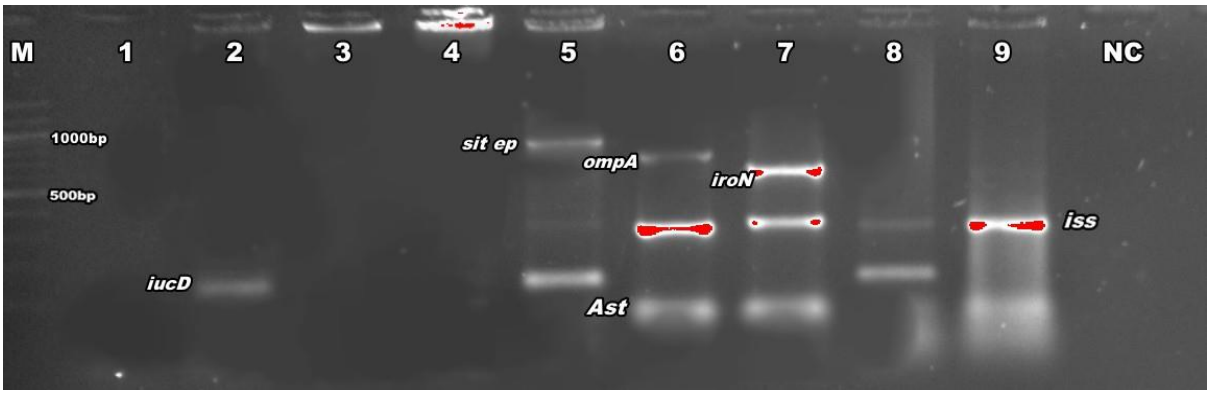

Figure 1: PCR detection of virulence genes *sit ep*(1032), *omp(919)A, iroN*(553), *astA*(116), *iss*(323) *and iucD*(269) NC is negative control. M is marker (100bp).

Out of the 16 virulence factors employed in this study, 12.5% of them were invasions (*ibeA* and *gimB*), 12.5% were adhesions (*papC* and *tsh*), 12.5% were toxins(*EAST-1* and *vat*), 37.5% were for iron acquisition (*chuA*, *OmpA*, *sit ep*, *iucD*, *iron* and *sit chr*) and the remaining 25% were for serum resistance (*iss*, *tra T*, and *cvi/cva*), (Table 3).

The most prevalent virulence genes detected were *tra T*, *iss* and *ibeA*; these were found in 62.3%,78.9% and 84.2% among APEC isolates and 30.6%, 24.9% and 20.2% among non-APEC strains respectively. The least virulence genes were *papC, cvi/cva, vat* each recording 10.5%, 5.3% and 10.5% among APEC isolates and 0.6%, 1,2% and 0.6% among non-APEC isolates respectively (figure 2).

The prevalence in the virulence genes between APEC and non-APEC were compared; of the 16 genes, 7 of the recorded a significant difference ($p<0.05$). These are *chuA, ibeA,traT, iroN, ompA, astA and irp2* (Table 3).

Table 3: Frequency of virulence genes by functional categories among APEC and other ExPEC *E.coli*

Virulence genes	Number (%) of *E. coli* isolates with virulence genes		p-value
	APEC n(19)	Non-APEC n(173)	
Iron Acquisition			
Chu A	10(52.6)	15(8.7)	0.0001*
Iro N			
Irp 2	4(21.1)	4(2.3)	0.0258*
IucD			
Sit Chr	2(10.5)	4(2.3)	0.0468*
Sit ep			

	5(26.3)	2(1.2)	0.1006
	1(5.3)	14(8.1)	0.0691
	4(21.1)	2(1.2)	0.1068
Serum Resistance			
Cvi/cva	1(5.3)	2(1.2)	0.2262
Iss			
Omp A	15(78.9)	43(24.9)	1
Tra T			
	4(21.1)	6(3.5)	0.0160*
Adhesins			
Pap c	12(62.3)	53(30.6)	0.0001*
Tsh			
Toxins			
Ast A	2(10.5)	1(0.6)	0.3714
Vat			
Invasins	2(10.5)	0(0)	-----
	9(47.4)	14(8.1)	0.0001*
	2(10.5)	1(0.6)	0.3714
Gim B	1(5.3)	7(4)	0.1312
Ibe A			
	16(84.2)	35(20.2)	0.0001*

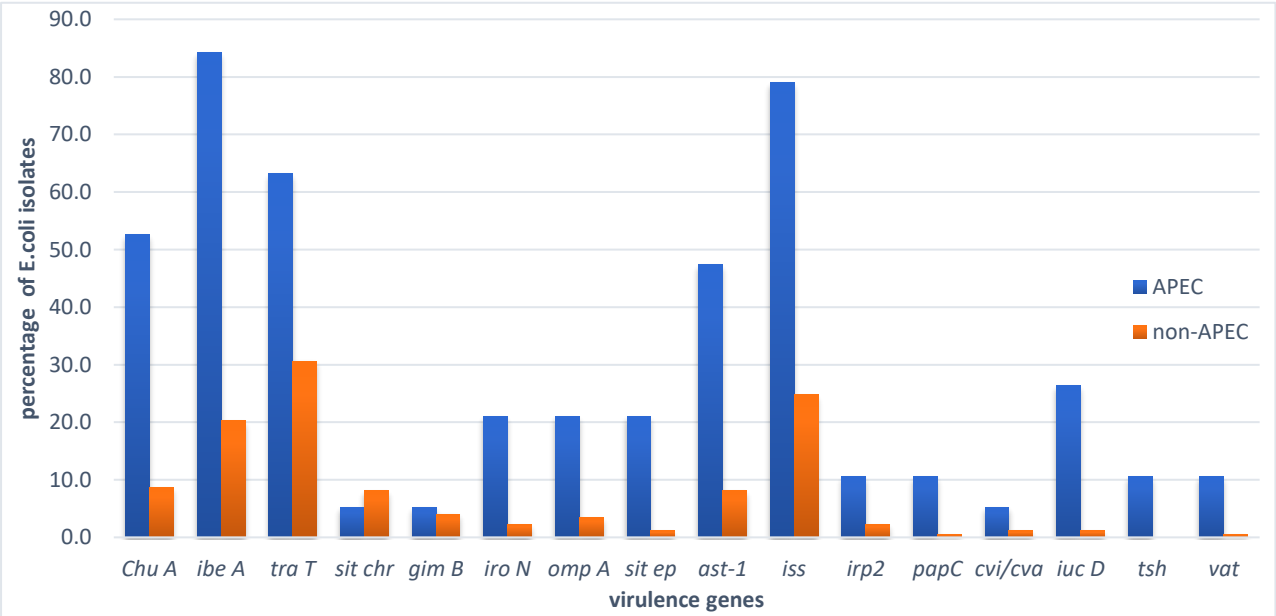

Figure 2: prevalence of virulence genes among APEC and non-APEC strains

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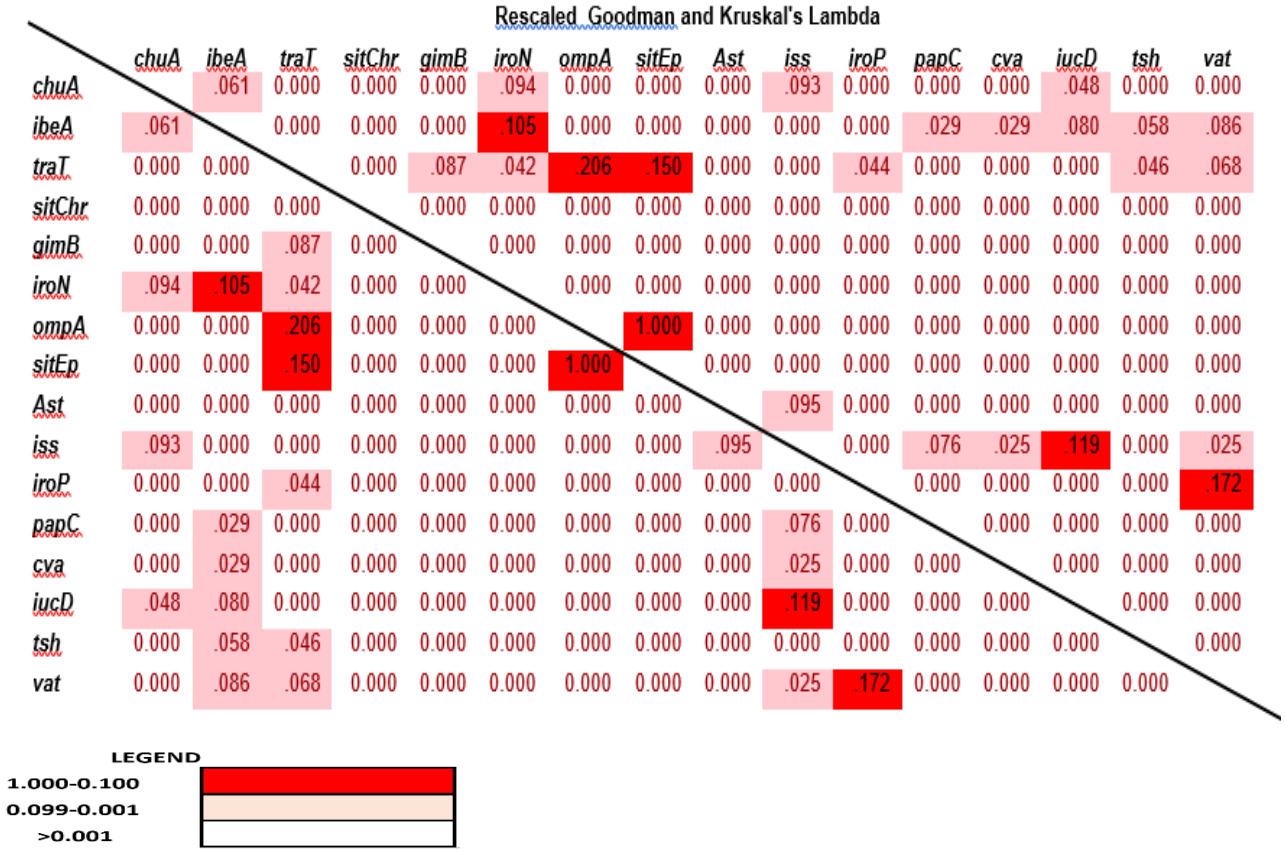

Figure 3: Statistical association between virulence genes of the *E. coli* isolates.

The statistical association between the various virulence genes were on figure 3. There were very strong associations were found between the genes *ibeA* and *iroN*, *sitEp* and *ompA*, *OmpA* and *tra T*, *tra T* and *sit Ep*, *irop* and *vat* and *iss* and *iucD* recording co-efficients of 1.05, 1.00, 0.206, 0.150, 0.172 and 0.119 respectively. Moderate associations, with co-efficient between 0.099 to 0.001 were observed among a number of pairs of virulence genes. These include *chuA* and *iroN*, *gimB* and *traT* and *astA* and *iss* with co-efficients of 0.094, 0.087 and 0.095 respectively. Weak associations, with coefficients≤0.001, occurred between the remaining virulence genes (figure 3).

Phylogroup profiling

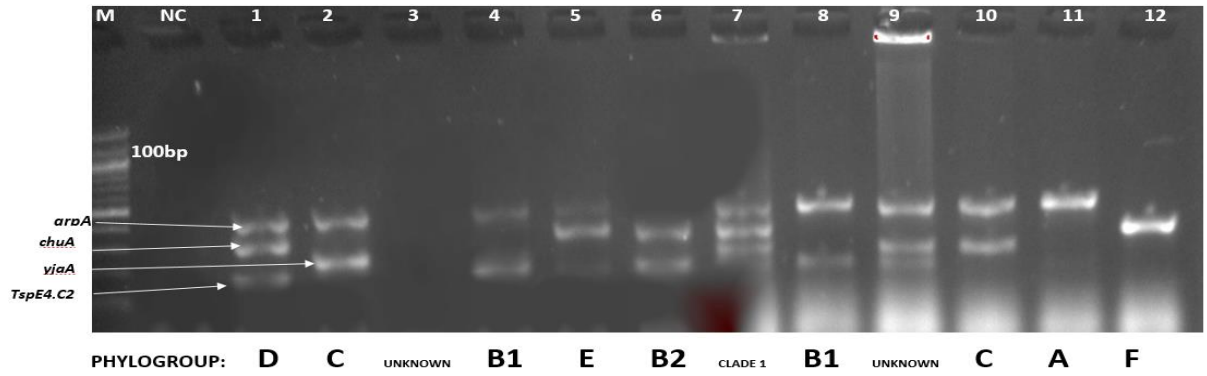

Figure 4: PCR detection of virulence genes *arpA, chuA, yjaA and TspE4.C2* NC is negative control. M is marker (100bp). The various combination of these genes and their corresponding phylo-groups: A, B1,B2,C,D,E,F, and clade 1

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Table 4: Distribution of virulence genes in extended phylogenetic structure among APEC isolates.

	Number of APEC isolates within phylogenetic groups							
VG category	A, 15.8% (n=3)	B1 10.5% (n=2)	B2 68.4% (n=13)	C 78.9% (n=15)	D 63.2% (n=12)	E 5.3% (n=1)	F 73.7% (n=14)	Clade 1 5.3% (1)

The 19 APEC isolates were screened with primers used in the quadruplex phylo-typing method listed in table 1 and their phylogenetic groups were determined base on the quadruplex genetypes profile described in table 2

Among the 8 phylogroups in the revised Clemont et al phylogrouping; group C recorded the highest number of APEC isolates, 78.9% and the least was clade 1 with 5.3% of the APEC isolates. Phylogroups F, B2, D, B1, E and A recorded 73.3%, 68.4%, 63.2%, 10.5%, 5.3% and 15.8% of the isolates respectively.

4. DISCUSSION

To the best of our knowledge, this research represents the very first time the revised Clemont phylo-genetic typing method have been used to examine the extended phylogenetic structure of *E. coli* amongst scavenging local chicken. Research of phylo-grouping among chicken have always targeted broilers. The research thereby gave us a deeper understanding of the genetic make-up of this population as well as their relation with virulence genes and can serve as a baseline studies for more researches into the pathogenicity of *E.coli* in scavenging local chicken.

Generally, our results showed that phylogroups D, B2, C and F are known to be associated with virulence strains whereas phylogroups A and B1 and E are known to be identified with commensal or less virulent strains**.** Among the APEC isolates, 63.2%, 68.4%, 78.9%, and 73.7% were found to belong to phylogroups D and B2, C and F respectively, whiles isolates in groups A, B1 and E were 15.8%,10.5% and 5.3% respectively (table 4). Several similar researches carried out revealed that, the frequency of phylogroups do vary from different geographical regions (Gordon and Cowling, 2003, Coura *et al.*, 2017). When Coura *et al*., 2017, investigated the phylo-groups in Tocantis state, central Brazil, they observed that phylo groups B2, C, D, E, and F are not common *E. coli* phylogroups isolated from poultry. Several reports from different geographical locations are consistent with their findings; Japan (Hiki *et al.*, 2014), Iran (Bagheri *et al.*, 2014) and Australia (Obeng *et al.*, 2012). All of them stressed particularly that phylogroups B1 and A are the most common among poultry. However, contradictory reports were made from several researches at different locations. In Egypt, Ramadan *et al.*, 2016 revealed a significantly higher occurrence of phylogroups D and B2. Similar researches where phylo-group D dominated includes: Italy (Pasquali *et al*., 2015), Canada (Aslam *et al*., 2014), China (Wang *et al*., 2010). These studies indicated that most highly virulent ExPEC strains belong to either group B2 or D. These strains have been known to harbor more virulence strains than those of A and B1 (Cortes *et al*. 2010, and Ramadam *et al*., 2016).

In majority of the above researches, the assigning phylo-groups were based on the old triplet method of phylo-grouping, but interestingly, their results were consistent with ours. This lays lays credence to an assertion by Lougue *et al.*, 2017; when the quadruplex system was applied, most isolates retain the same phylo-group they had in the old triplet method. In their study, Louge et al., (2017) observed that the expanded phylogenetic typing scheme was accurate and about 75% of isolates identified in the original typing scheme retained their original phylogroup.

The redistribution of the isolates in the new quadruplex system is associated with change in pathogenicity levels of the groups (Clemont *et al*., 2013, Logue *et al*., 2017). Phylo-group C is known to be originated from phylo group A, and phylogroup F from phylo group D Logue *et al.*, (2017). Emerging phylo-groups are known to be associated with higher virulent strains than their original groups (Logue *et al*., 2017).In line with this, 78.9% of the APEC isolates belong to phylo group C whiles a relatively lower (15.8%) belong to group A. Same redistribution occurred between F and D. F phylogroup habored more APEC isolates (73.7%) than the original D group (63.2%) (Table 4). This assertion was initially made by Logue *et al.*, (2017) when they conducted an extended phylo-typing on human and avian ExPEC and commensal *E. coli* Several studies confirmed this assertion (Bok *et al*., 2020). In their study, Lougue *et al.*, 2017 compared the previous and revised methods of phylo-grouping and realized that 75% of the isolates maintained their phylo-groups.

Horizontal transfer of the virulent genes occurs via mobile genetic elements; pathogencity island (PAI) and virulence plasmids. Analysis of association between the various virulence genes may identify which gene occur on the same genetic

element (Bonnet *et al*., 2009). A study of phylogenetic group assignment by content of virulence, resistance, replicon and pathogenicity island genes in APEC reveals that insertion of pathogenicity islands into the genome appears to correlate closely with revised phylogenetic assignment (Johnson *et al.*, 2006))

This research observed strong simultaneous occurrence of several iron acquisition genes with of structural gene for microcin *iss thus (iss/iuCD)*, outer membrane protein(*ompA*) thus (ompA/sitEp) (figure 3). As observed by Bonnet *et al*., 2009 and Johnson *et al*., 2006, these are indicative of the presence of virulence plasmid pAPEC-O2-ColV. Another plasmid of interested that showed its presence in this research is pAPEC-O2-R plasmid. It has been known to be associated with the complement resistance protein, *tra T* gene, and was originally isolated from an APEC strain (Johnson *et al*., 2005). In line with this, this gene was present in a higher percentage of APEC isolates than non-APEC.

Several virulence genes recorded significant ($P<0.05$) differences in their numbers between APEC and non-APEC strains. These can thereby be used as markers for APEC isolates identification. Among these, iron acquisition genes and serum resistance genes recorded the highest among isolates. Together, genes from these two groups were present in 80% of the isolates (table 3). Same conclusion was made by made by Paixao *et al*.(2016), they observed that virulence genes from these two groups were most prevalent amongst APEC strains.

An important virulence determinant for APEC strains is the ability to survive and grow in serum, where the concentration of free iron is extremely low. This also plays a role in the pathogenesis of colibacillosis (Gao *et al*., 2012). Thus, serum resistance is a vital factor to APEC survival. The low concentration of iron in the serum forces APEC to synthesize iron-uptake proteins which offers resistance to oxidative stress (Martinez *et al*., 2000; Janben *et al*., 2001). This study recorded significant differences between APEC and non-APEC isolates in the following iron-acquisition genes: *chuA*, *iroN* and *irp2*. Iron acquisition protects bacteria from host humoral immunity and the accumulation of these genes is a potential risk factor for APEC infection (Janben *et al*., 2001). The existence of several iron-related genes in APEC isolates is an indication that iron acquisition proteins play an important role in APEC pathogenicity, especially in sepsis-causing bacteria. Strong associations were identified between the genes within iron acquisition and serum resistance categories or between these two categories. The following pairs of genes; *ompA* and *iss, iucD and iss, ompA and sitEp, ompA and traT and traT and sitEp* are strongly associated, with co-efficient between 1.00 and 0.1 (figure 3). These genes usually occur together in the conserved virulence plasmidic (CVP) region, typical for the ExPEC virulence-associated plasmids. Earlier reports investigating the distribution of extra-intestinal pathogenic strains in these phylotypes confirmed this result. (Bonnet *et al*.2009 and Cortes *et al* 2010).

The toxin and invasion genes, *astA* and *ibeA* which are normally recorded in minimal percentages were found to be significant determinants of APEC in the study (figure 2). The *astA* and *ibeA* were significant ($p<0.001$, Table 3). All the literature reviewed and discussed in this study were of broilers because information of scavenging local chicken were very scant and virtually non-existent. We would then like to propose that there may be high correlation between APEC of SLC and toxins and invasion; a kind that doesn't exist in broilers and other fowl. Since this is the first line of research on APEC phylo-groups of SLC, it would be our recommendation that more researches are carried out on Avian *E. coli* surveillance with SLC as it reference point since it is one of the most consumed chicken in Tanzania

5. CONCLUSION

The research represent the first time the revised Clemont phylo-grouping system has been applied on scavenging local chicken in Tanzania. Among the APEC isolates, 63.2%, 68.4%, 78.9%, and 73.7% were found to belong to phylogroups D and B2, C and F respectively, whiles isolates in groups A, B1 and E were 15.8%,10.5% and 5.3% respectively. There was a strong simultaneous occurrence of several iron acquisition genes with of structural gene for microcin *iss thus (iss/iuCD)*, outer membrane protein(*ompA*) thus (ompA/sitEp). Iron acquisition genes and serum resistance genes recorded significant ($P<0.05$) differences between APEC and non-APEC strains and can thereby be used as markers for APEC isolates identification. The toxin and invasion genes, *astA* and *ibeA* which are normally recorded in minimal percentages were found to be significant determinants of APEC in the study.

Since this is the first line of research on APEC phylo-groups of SLC, it would be our recommendation that more researches are carried out on Avian *E. coli* surveillance with SLC as it reference point since it is one of the most consumed chicken in Tanzania

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APPENDIX - A

Table 1: List of primers used for virulence genes identification of APEC strains

Virulence factor	Target	Primer sequences	PCR product
Iron Aquisition	*Chu A(278)*	s: GACGAACCAACGGTCAGGAT a:TGCCGCCAGTACCAAAGACA	
	iro N 553	s: ATCCTCTGGTCGCTAACTG a:CTGCACTGGAAGAACTGTTCT	
	irp 2 (413)	s: AAGGATTCGCTGTTACCGGAC a:TCGTCGGGCAGCGTTTCTTCT	
	IucD(269)	s:ACAAAAAGTTCTATCGCTTCC a:CCTGATCCAGATGATGCTC	
	sit Chr(553)	s: ACTCCCATACACAGGATCTG a: CTGTCTGTGTCCGGAATGA	
	sit ep(1032)	s: TTGAGAACGACAGCGACTTC a: CTATCGAGCAGGTGAGGA	
Serum resistance	*cvi/cva (1181)*	s: TCCAAGCGGACCCCTTATAG a: CGCAGCATAGTTCCATGCT	
	Iss (323)	s: ATCACATAGGATTCTGCCG a:CAGCGGAGTATAGATGCCA	
	OmpA(918)	s: AGCTATCGCGATTGCAGTG a: GGTGTTGCCAGTAACCGG	
	tra T(430)	s: GTGGTGCGATGAGCACAG a:TAGTTCACATCTTCCACCATCG	
Adhesins	*pap C (501/328)*	s: TGATATCACGCAGTCAGTAGC a:CCGGCCATATTCACATAAC	
	Tsh (824)	s:ACTATTCTCTGCAGGAAGTC a:CTTCCGATGTTCTGAACGT	
Toxins	*ast A (116)*	s: TGCCATCAACACAGTATATCC a:TAGGATCCTCAGGTCGCGAGTGACG C	
	Vat(980)	s: TCCTGGGACATAATGGCTAG a: GTGTCAGAACGGAATTGTC	
Invasins	*GimB (736)*	s: TCCAGATTGAGCATATCCC a:CCTGTAACATGTTGGCTTCA	
	ibe A(341)	s: TGGAACCCGCTCGTAATATAC a:CTGCCTGTTCAAGCATTGCA	