Global Scientific JOURNALS

GSJ: Volume 9, Issue 4, April 2021, Online: ISSN 2320-9186
www.globalscientificjournal.com

ANTIBIOGRAM OF AVIAN PATHOGENIC *ESCHERICHIA COLI* IN SCAVENGING LOCAL CHICKEN IN MOROGORO , TANZANIA

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KeyWords
Ecoli, APEC, Virulence genes, susceptibility, antimicrobial, Morogoro ,Tanzania

ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) is responsible for the annual million-dollar loss in the poultry industry worldwide. This research aimed at investigating the occurrence and antimicrobial pattern of APEC among scavenging local chickens. A total of 400 cloacal and oropharyngeal swabs were obtained, out of which 192 *Escherichia coli* were isolated. By use of virulence factor profiling, these 192 samples were screened for the presence of 16 virulence factors by multiplex PCR. All 192 samples harbored at least one of the 16 virulence genes and 19 of them carried at least four, making them APEC. The virulence traits *ibeA, iss, traT and chuA* were observed to lead the chart with percentages of 84.21, 78.95, 63.16 and and 52.63 respectively. In the pathogenesis of APEC, Iron acquisition, serum resistance, toxins and invasins were found to be very significant ($P<0.05$). The antimicrobial sensitivity testing, 10.52% of the strains showed multi-drug resistance. All the isolates were sensitive to gentamycin and imipenem drugs whiles none of them were sensitive to cephalothin. Occurrence of virulence strains of APEC in Morogoro region of Tanzania is alarming.

INTRODUCTION

BACKGROUND INFORMATION

Selective pressure exerted by antimicrobials leads to the spread of multidrug resistance among avian *Escherichia coli* (19). Apparently-healthy poultry could harbor multidrug resistance of extra-intestinal *E. coli*. This presents a health risk to the main consumers, the human populace (25).

Scavenging local chickens makes over 70% of the entire chicken population in Tanzania). It has been observed that majority of these birds are kept mainly as free-ranged. A few of them, however, are housed as semi-intensive and a far lesser percentage of them are kept entirely intensive. This is hypothesized to have effect on the control of disease amongst them (17,26,32).

APEC and ExpEC strains from human hosts are also known to share similarities in contents of virulence genes and capacities to cause disease. This is because they both are known to encounter similar challenges in establishing infection in extra-intestinal locations (20).

PROBLEM STATEMENT AND STUDY JUSTIFICATION

PROBLEM STATEMENT

APEC infections lead to reduced yield, quality and hatching of eggs. It is responsible for the annual million-dollar loss in the poultry industry worldwide. It also causes *cellulitis* which is the second most leading cause of chicken morbidity and mortality (2,23)

JUSTIFICATION OF THE STUDY

Scavenging local chickens is heavily consumed in Tanzania and other developing countries leading to an increase in their demand (21,29). Since this poultry serve as the main host for APEC and the consumption of undercooked poultry may infect, which can serve as a reservoir for this pathotype, the potential for zoonotic transmission must be considered.

OBJECTIVES OF THE STUDY

MAIN OBJECTIVE

The main objective is to detect and determine the antibiogram of avian pathogenic *Escherichia coli* in scavenging local chicken in selected areas of Morogoro municipality, Tanzania.

THE SPECIFIC OBJECTIVES ARE:

i. To detect APEC strains among scavenging local chicken circulating in selected areas of Morogoro Municipality.

ii. To determine the antimicrobial susceptibility patterns of these APEC strains.

iii. To determine the occurrence and frequency of APEC virulence genes among the scavenging local chicken.

MATERIALS AND METHODS

STUDY DESIGN AND SAMPLE COLLECTION

$N= Z^2 P (1-P) /\Sigma^2$. N is sample size, Z is constant (1.96), P is prevalence and Σ is error margin (0.05). **200** samples were collected. This comprised 100 cloacae and 100 oral-pharynges swabs from each housing system; giving a total of **400** samples. This was a cross-sectional study; the samples were collected only once. The birds were not exposed to any pre-defined conditions.

Isolation of *Escherichia coli*

Procedures used were as described in the Bacterial Analytical Manual (BAM 2007). The organisms were grown on MacConkey and Blood Agar (OXOID, Hampshire, England) for primary isolation (5). The Blood agar base was supplemented with 10% horse blood. A loop full of each sample from the transport media was introduced on the media plate and was streaked appropriately with a sterile inoculating loop.

VIRULENCE FACTOR PROFILING TO DETECT APEC STRAINS

The positive *E. coli* strains, 192 in number, were investigated for various virulence genes by multiplex PCR (14). The procedures were performed in 25µl reaction mixtures. This included: 12.5 µl of Taq polymerase (Dream Taq PCR Master mix, Thermofischer thermoscientific 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 was 0.4 M. Conditions of the reaction mixtures included: 5mins at 95ºC initial denaturation, 94ºC of denaturation for 30s, annealing at 56ºC for 30s, elongation at 72ºC for 3minutes at 25 cycles, a final elongation at 72ºC for 10 minutes and a hold at 4ºC. A List of primers used is shown in the appendix.

ANTIMICROBIAL SUSCEPTIBILITY TEST OF APEC STRAINS

KIRBY-BAUER DISC DIFFUSION TEST

The Kirby-Bauer antimicrobial sensitivity test method was used to determine the isolates that were susceptible to cephalosporins and beta-lactams(3,6). Ten antimicrobial drugs were used. These included; augmentinin (30µg), imipenem (10µg), cephalothin (30µg), cefotaxime (30µg), ceftazadime (30µg), ceftriaxone (30µg), Nalidixic acid (30 µg), ciprofloxacin (5 µg), Gentamycin (10 µg) and Trimethoprim-sulphamethiozole (25 µg). The drugs were manufactured by Liofilchem limited, Italy.

ANALYSIS OF RESULTS

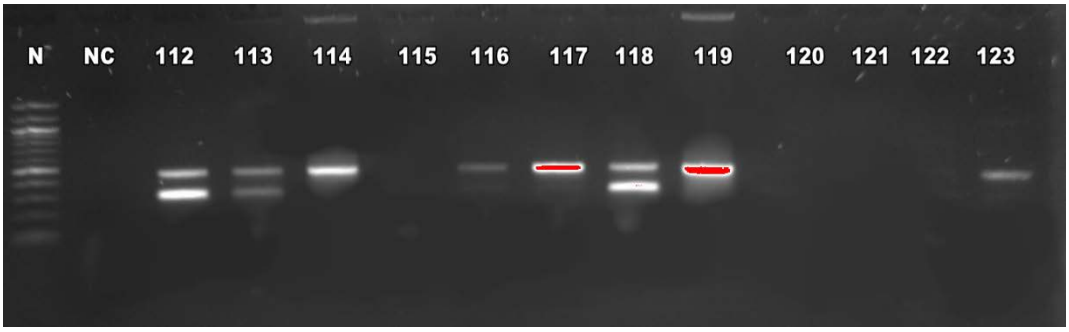
Biodata and laboratory data were analyzed using Epidemiological Package for Information (EPI Info) version 7 statistical software (CDC, Atlanta, GA, USA). The threshold for statistical significance was indicated in the table with a $P < 0.05$ reflected statistical significance.

RESULTS

Virulence factor profiling to detect APEC strains

After primary culture of samples on Macconkey and blood agar; 192, out of 400 samples were positive for *Escherichia coli.* The suspected positive *Escherichia coli* isolates were all confirmed in the biochemical tests. PCR amplification to detect the virulence genes showed that 19 out of 192 samples, (9.8%) were APEC positive(fig 1). Thus, these isolate had at least 4 virulence factors.

Figure 1



Note:PCR detection of virulence of genes of APEC (*traT and chuA*). Multiplex PCR visualized under gel documentation. Lanes 112,113,114,116,117,118,119 and 123 are positive for gene *traT* (430bp) while lane 112,113 and 118 are positive for gene *chuA*(278bp). NC is negative control. M is marker (100bp)

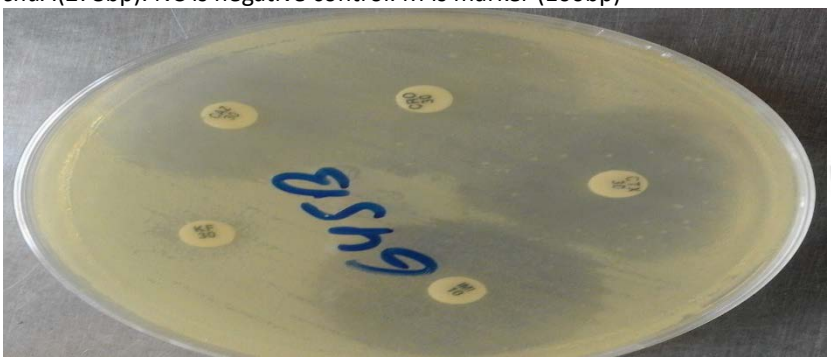


Fig 2: Sensitivity test for sample with antimicrobial discs ciprofloxacin (KF), ceftazadime (CAZ), ceftriaxone (CRO), ceftriaxone (CTX), . The test isolate is sensitive to all listed drugs except ciprofloxacin (KF)

Table 1 Prevalence of virulence genes among APEC strains

Virulence factor	Number of APEC isolates that harbored gene (N=19)	Percentage	P-value Percentage
Iron Acquisition			
chu A	10	52.63	52.63
iro N	4	21.05	21.05
irp 2	2	10.53	10.53
iucD	5	26.32	26.32
sit Chr	1	5.26	5.26

sit ep	4	21.05	0.016	21.05
Serum resistance				
cvi/cva	1	5.26		5.26
iss	15	78.95		78.95
omp A	4	21.05		21.05
tra T	12	63.16	0.030	63.16
Adhesins				
pap C	2	10.53		10.53
tsh	2	10.53	0.602	10.53
Toxins				
ast A	9	47.37		47.37
vat	2	10.53	0.005	10.53
Invasins				
gimB	1	5.26		5.26
ibe A	16	84.21	0.00001	84.21

ANTIMICROBIAL SUSCEPTIBILITY

KIRBY-BAUER DISC DIFFUSION TEST

The antimicrobial phenotypes of APEC isolates were investigated using Kirby-Bauer disc diffusion method(fig 2). The zones of inhibition were measured and the resistance was recorded based on Clinical and Laboratory Standards Institute (CLSI).

Table 2: Proportion of antimicrobial resistance profile of APEC strains

	Susceptible isolates		Intermediate isolates		Resistant isolates	
Antimicrobial	**Number**	**Percent**	**Number**	**Percent**	**Number**	**Percent**
CRO(30µg)	14	73.68	3	15.79	2	11.78
CTX(30µg)	12	63.16	4	21.05	3	17.65
CAZ(30µg)	15	78.95	3	15.79	1	5.88
CN(10µg)	19	100	0	0	0	0
STX(25µg)	8	42.11	0	0	11	64.71
AUG(30µg)	16	84.21	0	0	3	17.65
NA(30µg)	12	63.16	0	0	7	41.18
CIP(5µg)	10	52.63	9	47.37	0	0
KF(30µg)	3	15.79	2	10.53	14	82.35
IMI(10µg)	19	100	0	0	0	0

Note: Antimicrobial resistance profiles of APEC. CRO: ceftriaxone, CTX: cefotaxime, CAZ: ceftazadime, CN: gentamycin, STX: Trimethroprim-Sulfamethoxazole, AUG: augmentin, NA: nalidixic acid, CIP: ciprofloxacin KF: cephalothin, IMI: imipenem.
Resistance of isolates were high to cephalothin and sulphonamide drugs while more isolates were susceptible to imipenem, augmentin, ceftriaxone and ceftazidime drugs.

DISCUSSION

All single virulence genes that are found in any APEC strains are present in all non-pathogenic strains as well, thus different virulence mechanisms are employed by different putative pathotypes as observed in earlier researches (12, 23). Although samples were collected from apparently healthy chickens, at least one virulence factor was present in all 192 *E. coli* isolates. This supports the notion that commensal *E. coli* in the intestines of healthy birds may carry an array of virulence factors of APEC that makes them potentially pathogenic (7).

Iron acquisition, serum resistance, toxins and invasion were seen to be statistically significant to the pathogenesis of APEC, with P-values < 0.05 (table 1). The most widely distributed virulence gene is *ibeA* (invasion of the brain epithelium), which was harbored by 84.21 % of the isolates (table 1). Its association with the pathogenecity of avian ExPEC strains for chicken was investigated and it was known to increase in early stage of chicken infection (8,37). Thus, majority of these chickens may be at risk of any opportunistic disease that may break.

The second most prevalent factor noted is the *iss* (increased serum survival) gene which was harbored by 78.95% of the isolates (Table 1). A total of 61 *E. coli* isolates from chicken flocks with respiratory symptomatology was probed for the presence of seven virulence genes(31). The *iss* gene was harbored by 73.8% of the chicken, the highest amongst the seven. Although their results correspond to one observed in this study, we took samples from apparently healthy chicken while they isolated samples from diseased birds. This gives an indication that apparently healthy chickens could also be a repository of virulence genes, especially *iss*.

In reference to iron acquisition virulence factors, the *chuA* gene was the highest (table 1). It specifically encodes an outer-memberane protein that is involved in heme uptake in *E .coli*. It's been known to be a very significant iron acquisition factor (33). It was harbored by 52.63% of the isolates.

Toxins of APEC were amongst the least recorded. The *vat* and *astA* genes were harbored by 10.53% and 47.37% of the APEC isolates respectively (table 1). Similar observations were made in a longitudinal study to explore the carriage of virulence-associated genes by chicken (22). They realized that toxin encoding genes of APEC were the least frequently detected. In their study, the *vat* and *astA* genes averaged 2.11% and 1.12% prevalence respectively. In a different study, it was noted that the *astA* gene product is an enteroaggregative heat-stable toxin called EAST 1. They also recorded low prevalence of these APEC toxins in comparison with other virulence genes when they assessed the genetic similarity between APEC and *E. coli* strains in healthy birds (30).

Adhesins are being reported to have low incidence in avian pathogenic isolates; as such they are not reliable tools for diagnosis (34).

It is therefore not surprising that this study observes them as amongst the least virulence factors; *Pap C* and *Tsh* genes each present in 10.53% of the isolates.

In antimicrobial sensitivity test, as high as 94.7% of the APEC strains showed resistance to at least one of the antimicrobial tested against (table 2). In one of the earliest researches to investigate antimicrobial resistance phenotypes in *E. coli* isolates from scavenging local chicken in Tanzania, it was noted that 87.01% of the birds showed resistance to at least one of the drugs (24). Two years down the line, an increase to 94.7% shows a severe and alarming incidence of antimicrobial resistance in scavenging local chicken

An isolate is said to be multi-drug resistant (MDR) if it resists drugs from three or more categories. In a research to characterized APEC strains isolated from poultry and realized that 92% of the strains presented MDR with the highest resistance to sulfamethoxazole (9). In this study, 10.52% of APEC isolates were MDR, hence calling for concern (table 2). Also sulfamethoxazole is one of the least active drugs, with 64.71% of the isolates being resistant to it.

All isolates were sensitive to gentamycin and imipenem, an indication that these drugs are the most active antimicrobials. Aminoglycosides, particularly gentamicin are the most effective antimicrobials against isolated bacteria. These drugs are rarely used in the treatment of human or veterinary-associated illnesses due to their high cost. As such, there is no selective pressure on these drugs among the bacteria and hence bacteria are very sensitive to them.

The least active drug observed was cephalothin. As high as 82.35% of the isolates resisted it (table 2). This was anticipated because it is a first generation cephalosporin and has been known for its prophalytic use in animals for years. As such, it is the weakest cephalosporin (15).

CONCLUSIONS

Single virulence genes observed among APEC strains were also present among the commensal *E. coli* as well, thus APEC is now considered a primary pathogen rather than a result of respiratory or immune-suppressive viral illnesses. Clinically healthy local scavenging chickens may act as reservoirs for APEC.

Majority of the APEC strains harbored *ibeA, iss*, *traT* and *chuA* with percentages of 84.21, 78.95, 63.16 and 52.63 respectively. Also thus invasions, serum resistance and iron acquisition systems and toxins play significant roles in the pathogenecity of APEC ($P<0.05$).

With regard to antimicrobial resistance, this research concludes that it is very much on the increase. As high as 94.7% of the APEC isolates showed resistance to at least one of the antimicrobials and 10.5% of them were multi-drug resistant. All the isolates were sensitive to gentamycin and imipenem drugs whiles none of them were sensitive to cephalothin.

Acknowledgment

The authors wish to Prof Gerald Misinzo for his encouragement and support. Special thanks also goes to Mr Mugusi and Mr Hashim, all of the Department of Microbiology, Parasitology and Immunology, College of Veterinary and Medical Sciences, Sokoine University of Agriculture, Morogoro, Tanzania. The scholarship for the Masters Degree study which produced this work was by Intra ACP Mobility scheme.

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