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Characterization of Multidrug Resistant *Escherichia coli* Isolates Recovered from Humans and Chickens, Trinidad and Tobago

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Abstract

To characterize extended-spectrum beta-lactamase (ESBL) and extra-intestinal pathogenic *Escherichia coli* (ExPEC) associated virulence genes in *E. coli* isolates from chickens and humans in Trinidad and Tobago. This cross sectional study was conducted over a three-month period. A total of 471 *E. coli* isolates; 160 from humans treated at a regional tertiary hospital and 311 from chicken caecal samples from "pluck shops" in Trinidad & Tobago were identified using both conventional and molecular microbiological methods. Phenotypic confirmation of ESBL producing *E. coli* isolates from humans was by Microscan system (Siemens, USA) while the double disk diffusion method was used for the chicken isolates. Polymerase chain reaction (PCR) analysis was used to determine the ESBL and ExPEC-associated virulence genes in representative human isolates and all chicken isolates. From the 311 chicken *E. coli* isolates, 49.2% (153/311) produced ESBL, while 56.3% (90/160) from humans were ESBL positive. All human and chicken ESBL isolates were 100% susceptible to carbapenems and aminoglycosides antimicrobials. PCR detected 21.1% bla_{CTX-M}, 13.3% bla_{TEM} and 7.8% bla_{SHV} genes among *E coli* isolates from humans compared to 0.6% bla_{CTX-M} and 48.6% bla_{TEM} genes in chickens. PCR analysis revealed diverse virulence profiles among the isolates. There was a high occurrence rate of ExPEC-associated virulence genes in *E. coli* isolates from both humans and chickens. However, the *CTX-M*-1 genes were most predominant in humans while *TEM* occurred in chicken isolates. The diverse ESBL and virulence associated gene profiles encountered in *E. coli* isolates from humans and chickens on the surface depicts no similarity or relationships despite occurrence in both cohort groups. Therefore *E. coli* strains from chickens and humans require further investigation to determine their clonal relatedness or transmission in the country.

*Deceased.

1. Introduction

Escherichia coli are common inhabitants of the gastrointestinal tract of animals and humans which exist in pathogenic and nonpathogenic (commensal) forms. Majority of pathogenic strains cause intestinal-related illnesses, but a few are implicated in extra-intestinal infections in either animals or humans hence are designated as extra-intestinal pathogenic *Escherichia coli* (exPEC). ExPEC strains are further sub-divided into avian pathogenic *Escherichia coli* (APEC), which cause a systemic disease in avian species, neonatal meningitis-causing *Escherichia coli* (NMEC) and uropathogenic *Escherichia coli* (UPEC) responsible for neonatal meningitis and urinary tract infections in humans respectively. Comparative genomic studies have shown a high degree of relatedness between these ExPEC subsets [1] [2].

Published reports suggest that poultry may be a reservoir of strains causing disease in humans [3] [4] [5]. An earlier study reported that women succumbing to urinary tract infections caused by multidrug-resistant *E. coli* were significantly associated with frequent consumption of chicken products [6], providing the first direct linkage between chicken and human ExPEC infections. Subsequent studies have reported that food and chickens serve as a reservoir for *E. coli* causing urinary tract infections in humans [7] [8]. Poultry products (especially meat and eggs) are the most consumed protein sources in Trinidad, thus increasing the likelihood of direct or indirect transmission of bacterial pathogens from poultry to humans. The risk of transmission is further increased through rearing of chickens in the backyard further exposing many families to direct or indirect contact with chickens or their waste. Direct transmission of antibiotic-resistant *E. coli* from poultry to humans has indeed been reported in literature [9] [10]. Recent studies have shown a high degree of genetic similarities between *E. coli* strains from chicken meat, poultry and human extra-intestinal infections [11] [12] [13] [14] [15], suggesting a zoonotic transmission of such strains.

The rising global emergence of multi-drug resistant bacteria negates significant advances gained in the treatment of bacterial diseases in modern medicine. This emergence of multidrug resistant bacterial strains which has presented antibiotic resistance crisis is believed to be linked to extensive or uncontrolled use of antimicrobials in agriculture [16]. An increasingly recognized mechanism of resistance to antibiotics mediating multidrug resistance in Gram-negative bacteria including *E. coli*, is the production of β-lactamases, especially extended-spectrum β-lactamases (ESBLs) and *AmpC* [17] and recently, the New Dehli Metallo-beta-lactamase-1 (NDM-1) has been reported [18].

Although extensive research has been conducted elsewhere on the prevalence of ESBLs and virulence-associated genes in ExPEC strains from humans and poultry [7]

[19] [20], there is still a dearth of information in the Caribbean region. Although ESBL-producing bacteria including *E. coli* strains from humans in Trinidad & Tobago have previously been reported [21] [22], to date, there has not been any published report on a comparative analysis of virulence-associated genes in *E. coli* isolates from humans and those from poultry especially chickens. Therefore, the current study examined if a relationship exists between *E. coli* strains from extra-intestinal infections in humans and those from chickens entering the human food pathway by characterizing ESBL subtypes and profiles of ExPEC-associated virulence genes.

2. Materials and Methods

2.1. Study Design

This cross-sectional study was conducted over a three-month period (March-May, 2014) to characterize *Escherichia coli* isolates from humans and chickens in Trinidad and Tobago.

2.2. Bacterial Isolates from Humans

Human bacterial isolates were recovered from routine clinical specimens (blood n = 60, Cerebrospinal fluid CSF n = 12, wound swabs n = 94, urine n = 80 and sputum n = 35). These were processed using standard microbiological procedures (including Gram stain, culture with appropriate media, biochemical tests—catalase, coagulase, oxidase, sugar tests etc.) to conventionally identify the *E. coli* isolates [24]. These isolates were further analyzed with the automated microdilution machine, MicroScan WalkAway-96 System (Siemens, USA) to confirm the species and determine their minimum inhibitory concentration (MIC) values at concentrations and breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial susceptibility and ESBL screening [25]. All isolates primarily identified by the MicroScan system as ESBL producers with increased MIC breakpoints interpreted as resistant, and observed to be multidrug resistant (resistant to three or more class of antibiotics) were included in the analysis.

2.3. Bacterial Isolates from Chickens

Ceca of chicken samples were obtained and cultured for the isolation of *E. coli*. Isolates of *E. coli* were recovered from the samples using the following protocol. "Pluck shops", defined as a poultry processor operating from a fixed location where live poultry is converted into raw poultry products fit for human consumption, surrounding the tertiary hospital were targeted for convenience and sampling was carried out on separate occasions. Fresh chicken guts were collected from eight "pluck shops" along the East-West corridor of Trinidad and placed in sterile sampling bags. All samples (n = 327) were transported to the microbiology laboratory at the School of Veterinary Science, The University of the West Indies, St. Augustine Campus in chilled containers and analysis carried out within 4 h of collection. The cecal sacs were separated from the interstitial material. They were aseptically opened using alcohol-sterilized scissors and an

opening of 1 - 2 cm in diameter was made at the top wall of the cecum. Using sterile tweezers, fecal materials from the cecum were placed on MacConkey agar (Oxoid, Basingstoke, UK), ensuring as much as possible that the internal surface area of the cecum did not make contact with the agar. The inoculum was then streaked out directly on MacConkey and Blood agar plates. All plates were incubated at 37˚C for 18 - 24 h and examined for bacterial colonies. Suspected *E. coli* isolates were then identified using standard methods [24]. In all 311 bacterial isolates from the 327 chicken samples were confirmed as E. coli and were included in the final analysis.

2.4. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) of *E. coli* isolates from chickens were determined by modified Kirby Bauer disk diffusion on Muller Hinton (MH) agar against 15 antimicrobial agents according to CLSI guidelines [25]. The following antibiotics discs and concentrations were used: amoxy/clavulanic acid (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefoxitin (30 μg), cefpodoxime (10 μg), entapenem (10 μg), imipenem (10 μg), meropenem (10 μg) ciprofloxacin (5 μg), norfloxacin(10 μg), Amikacin, (30 μg), sulphamethoxazole/trimethoprim (25 μg), gentamicin (10 μg) and tetracycline (30 μg) (all from Oxoid, Basingstoke, UK). To determine the susceptibility of the *E. coli* isolates to the antimicrobial agents, pure colony were suspended in phosphate-buffer to a 0.5 McFarland turbidity standard and then streaked on MH agar plates. Excess inoculum was aseptically removed, and the agar plates allowed to dry before dispensing antibiotic discs. After overnight incubation at 35˚C temperature, plates were examined for zones of inhibition and diameters were measured using a caliper. Interpretation of the zones of inhibition was done as stipulated by the Clinical and Laboratory Standards Institute (CLSI, 2014). *Escherichia coli* ATCC 25,922 strain was used for quality control.

2.5. Confirmation of ESBL Phenotypes in Chicken Isolates

ESBL phenotypes were confirmed by using 30 μg amoxicillin-clavulanic acid disc surrounded by discs of 30 μg ceftazidime, cefotaxime and cefotixin at a distance of 15 mm on MH agar plate inoculated according to standard procedures and CLSI guidelines [25]. After overnight incubation at 37˚C, any enhancement of the zone of inhibition between a β-lactam disc and a disc containing the β-lactamase inhibitor was indicative of the presence of ESBL.

The following CLSI ESBL screening cut-off values were used for third-generation cephalosporins: cefpodoxime (10 μg/disk) ≤ 17 mm; ceftazidime (30 μg/disk) ≤ 17 mm; cefotaxime (30 μg/disk) ≤ 22 mm; and ceftriaxone (30 μg/disk) ≤ 19 mm [25]. *E. coli* ATCC 25,922 strain was used as a negative ESBL control. Each confirmed isolate by these conventional microbiological tests was stored in brain heart infusion broth with 15% (v/v) glycerol at −20˚C until subjected to further molecular analysis.

2.6. Molecular Analysis

All *E. coli* isolates from chicken (n = 311) were subjected to molecular analysis for

ESBL genes while only multidrug resistant phenotypically confirmed as ESBL producers by the automated system in humans (n = 90) were only subjected to molecular analysis for the presence of the ESBL genes: *TEM*, *SHV*, *CTX-M* group 1 & 2, *CMY*-2 and *AmpC* using PCR tool as previously described [20]. Oligonucleotide primers used in this study and their respective annealing temperatures are listed in Table 1. The reaction mixture consisted of the following: 12.5 μl GoTaq® Green Master Mix (Pro-

Table 1. Oligonucleotide primers used in the analysis of ESBLs and virulence-associated genes in Escherichia coli isolates from humans and chickens, Trinidad and Tobago.

Gene	Primer name	Sequence (5' to 3')	Annealing T (˚C)	Reference
ESBLs				
bla_{SHV}	SHV-F	TTATCTCCCTGTTAGCCACC	58	[19]
	SHV-R	GATTTGCTGATTTCGCTCGG		
bla_{TEM}	TEM-F	GCGGAACCCCTATTTG	55	[19]
	TEM-R	ACCAATGCTTAATCAGTGAG		
bla_{CTX-M1}	CTX-M 1-F	GGTTAAAAAATCACTGCGTC	56	[19]
	CTX-M1-R	TTGGTGACGATTTTAGCCGC		
bla_{CTX-M2}	CTX-M2-F	GATGAGACCTTCCGTCTGGA	61	[19]
	CTX-M2-R	CAGAAACCGTGGGTTACGAT		
bla_{CMY-2}	CMY-2 F	ATGATGAAAAAATCGTTATGCTGC	62	[19]
	CMY-2 R	GCTTTTCAAGAATGCGCCAGG		
AmpC	AmpC-F	ATGATGAAAAAATCGTTATGC	56	[19]
	AmpC-R	TTGCAGCTTTTCAAGAATGCGC		
ExPEC-associated virulence genes				
fimC	*fimC-F*	TAAGGAAATCGCAGGAA	52.0	[20]
	fimC-R	GTCAGGTAATAGGGTGTCG		
ompA	*ompA-F*	TTTTGGATGATAACGAGG	50.0	[20]
	ompA-R	TGCTGGGTAAGGAATAAC		
betA	*betA-F*	CGGTTTCGGCGTGGTTAC	60.0	[20]
	betA-R	GTGGGCATGTGCGGATTA		
ibeA	*ibeA-F*	AGGCAGGTGTGCGCCGCGTAC	58.0	[20]
	ibeA-R	TGGTGCTCCGGCAAACCATGC		
rfaH	*rfaH-F*	TGTATCGCAACACGCACG	54.0	[20]
	rfaH-R	AAAACCAGTCGAGCCTTC		
irp2	*irp2-F*	AAGGATTGCCTGTTACCGGAC	52.0	[20]
	irp2-R	TCGTCGGGCAGCGTTTCTTCT		
fyuA	*fyuA-F*	GCTTTATCCTCTGGCCTT	52.0	[20]
	fyuA-R	GGCATATTGACGATTAAC		

T = Temperature; Reference = Reference cited.

mega, Madison, WI), 2 μl of each primer (forward and reverse) and small portion of colony as source of template DNA, made to 25 μl with nuclease-free water. The PCR cycling reaction comprised the following: an initial denaturation step at 94˚C for 5 min, followed by 25 cycles of 94˚C for 30 sec, annealing at specified temperature for 45 s, extension for 1 min and then a single final extension at 72˚C for 5 min. Amplicons (5 μl) were resolved on 1% agarose gel in 1× Tris-Acetate-EDTA (TAE) buffer alongside a 100 bp ladder (New England Biolabs, Ipswich, MA) and viewed on a UV trans-illuminator after staining with Gelred™ dye (Biotium Inc., Hayward, CA). The validity of the representative amplicons was ascertained by DNA sequencing. Resolved amplicons were purified of agarose gel using a Qiagen gel extraction kit, cloned into TOPO vector (Life Technologies, Sao Paulo, Brazil) and then submitted to Macrogen Korea (Seoul, South Korea) for sequencing. Sequences were confirmed with BLAST analysis at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

2.7. Virulence Genotyping

Representative ESBL-producing *E. coli* isolates from both humans (n = 42) and chickens (n = 45) were subjected to PCR analysis for a panel of ExPEC-associated virulence genes as previously reported with slight modifications [3] [19] [20]. These genes include: *irp*2 (iron-repressible protein), *fyu*A (yersiniabactin synthesis), *ibe*A (invasion of brain endothelium), *bet*A (choline dehydrogenase), *rfa*H (a transcriptional anti-terminator), *fim*C (type 1 fimbriae) *and Omp*A (outer membrane protein A). Oligonucleotide primers used in this study, target genes and their respective annealing temperatures are listed in Table 1. PCR amplification and subsequent detection of amplicons were performed as described above. A 100 bp DNA ladder was used as a molecular size marker.

2.8. Statistical Analysis

Frequencies of ESBL and ExPEC-associated virulence genes were calculated using Epi info™ and Microsoft Excel. Chi-squared test and Fisher's exact test were used as appropriate to compare data from different groups. Data were reported as comparisons of frequency distributions and a p-value < 0.05 was considered statistically significant.

2.9. Ethical Approval

The study was approved by the Ethics Committee of the University of the West Indies, St. Augustine Campus.

3. Results

A total of 471 *E. coli* isolates (humans n = 160, chickens n = 311) were included in the final analysis. Although not shown, the antimicrobial susceptibility of *E. coli* isolates from both humans and chickens revealed that all (100%) were susceptible to tested carbapenems and aminoglycosides agents. *E. coli* isolates from humans (54%) were resistant to tested cephalosporins while cefotixin showed the highest susceptibility (78%), the least was cefotaxime (40%). Isolates from chickens on the other hand exhibited over

95% susceptibility to all the cephalosporins. There was resistance (>45%) in amoxycillin/clavulanate, tetracyclines, fluoroquinolones and sulphamethoxazole/trimethoprim in *E. coli* isolates from both humans and chickens.

More than half of analyzed isolates 51.6% (243/471) were ESBL producers (humans 56.2%, 90/160; chickens 49.2%, 153/311; p = 0.8). As depicted in Table 2, the predominant ESBL gene encountered among *E. coli* isolates recovered from chickens was 48.6% *TEM* compared to 13.3% from humans (p < 0.01). No single *CMY-2*, *SHV*, *AmpC* or even their combined genes was recovered from *E. coli* isolates from the chickens. Despite the high frequency of *TEM* ESBL genes among *E. coli* isolates from the chickens, there was only a paltry 0.6% occurrence of CTX-M-1 gene. All ESBL genes assayed for were not detected in 50.8% and 11.1% of isolates from chickens and humans, respectively. The difference was statistically significant (p < 0.01).

E. coli isolates with co-existing CTX-M-1 and CTX-M-2 genes, tested negative for all the virulence genes among chicken isolates. This pattern was also observed in the isolate with the *CMY-2* gene only. The frequency of distribution of ExPEC-associated virulence genes in human and chicken isolates are shown in Table 3. All *TEM*-positives *E. coli* isolates from chicken samples lacked the *irp*2 gene which was predominant in isolates from humans. *ibeA* was also absent in isolates from chickens, but was present

Table 2. Distribution of genes among ESBL-producing *E. coli* isolates recovered from humans and chickens, Trinidad and Tobago (%).

bla genes	Humans (n = 90)	Chickens (n = 311)
TEM	12 (13.3)	151(48.6)
CTX-M-1	19 (21.1)	2 (0.6)
CMY-2	6 (6.7)	0 (0.0)
SHV	7 (7.8)	0 (0.0)
CTX-M-1 CTX-M-2	6 (6.7)	0 (0.0)
CMY-2 AmpC	5 (5.5)	0 (0.0)
TEM CTX-M-1	25 (27.8)	0 (0.0)
NEGATIVE	10 (11.1)	158 (50.8)

Table 3. Occurrence of virulence genes in representative ESBL-producing *E. coli* isolates recovered from humans and chickens, Trinidad and Tobago (%).

Virulence factor genes	Humans (n = 42)	Chickens (n = 45)	p-value
fimC	27 (64.3)	27 (60.0)	1.000
ompA	33 (78.6)	45 (100.0)	1.000
rfaH	36 (85.7)	6 (13.3)	0.002
irp2	33 (78.6)	0 (0.0)	<0.01
betA	36 (85.7)	36 (80.0)	1.000
fyuA	33 (78.6)	12 (26.7)	0.002
ibeA	9 (21.4)	0 (0.0)	<0.01

in nine isolates from humans harboring *TEM*, *CTX*-M1, *CMY*-2 and *AmpC* genes.

The gene *ompA* was the most frequent (100%) virulence gene in chicken isolates, followed by *betA* (80%) and *fimC* (60%). None of the chicken isolates possessed *irp*2 and *ibeA*. In contrast, both *rfaH* and *betA* had distribution frequency of 85.7%, followed by *irp*2, *fyuA* and *ompA* at 78.6%, *fimC* (64.3%) and *ibeA* (21.4%). Genes *rfaH*, *irp*2, *fyuA* and *ibeA* were detected at statistically significantly higher frequencies in isolates from human isolates than ones from chicken (p < 0.05). The occurrences of *betA*, *fimC*, and *ompA* were similar between the human and chicken isolates, whereas *irp*2 and *ibeA* genes were only detected in human isolates.
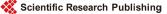
4. Discussions

This study was designed to characterize the ESBL subtypes and profiles of ExPEC associated virulence genes among *E. coli* strains from humans and those from chickens that could enter the human food pathway in Trinidad and Tobago.

The antimicrobial susceptibility of each group (humans and chickens) revealed that all were susceptible to tested carbapenems and aminoglycosides agents. This is an important finding in the country because the option still exists for use of these class of antimicrobial agents in both humans and agricultural health sectors. Most *E. coli* isolates from humans were resistant to more than half of the tested cephalosporins (54%) while those from chickens exhibited over 95% susceptibility to all the cephalosporins. This does not seem to support any evidence of transfer from chickens to humans which again is suggestive of the absence of zoonotic spread *E. coli* strains resistance to this class of antimicrobial agents between chickens and humans in the country [26].

Generally it was observed that *E. coli* isolates recovered from humans (88.9%) carried more of the ESBL genes than those from chickens (49.2%), again suggesting a less likelihood of transfer of such genes from chickens to humans in the country.

The *TEM* genes in this analysis were observed to be predominant among *E. coli* isolates from chicken (48.6%) as opposed to the frequency in isolates from humans (13%). A similar predominance of *TEM* genes has also been reported in China and Belgium [27] [28] [29] among *E. coli* isolates from both chickens and humans. *TEM* is the most common ESBL found worldwide, and therefore its occurrence in chicken isolates was not surprising. Although the *TEM* genes are not classified as ESBL but they have been noted to confer ESBL properties [30].

Another major finding was that *CTX-M*-1 was the most prevalent among ESBL-producing isolates from humans and this is in agreement with a previous study conducted in this institution [23]. Such a high predominance of *CTX-M* group 1 alleles has also been previously reported among clinical isolates elsewhere [31]. In this current study, there appears to be no likelihood of acquisition of the CTX-M group genes from *E. coli* isolates recovered from chickens since the frequency of these genes was very low or nonexistent in the chicken samples. This is in contrast to reports elsewhere where these have been recovered [32] [33]. Further studies however need to be carried out to determine if there was any clonal similarity or relatedness between the few that was

seen in the chicken isolates with ones from humans.

Except for *ibeA* and *irp*2 genes that were not detected among the *E. coli* isolates from chicken samples in this study, all the other ExPEC-associated virulence genes were detected in high frequencies in isolates from both humans and chicken specimens. These ExPEC-associated virulence genes were selected on the basis of having been previously used in studying isolates from poultry and humans; and also of being known to be located on high pathogenicity island of *E. coli* strains that are pathogenic to humans [19] [20] [34]. The functions and distribution frequencies of other ExPEC-associated virulence genes in poultry *E. coli* isolates have previously been reported in literature [34] [35]. The high rate of detection of these genetic profiles especially the *fimC*, *ompA*, *betA and fyuA* observed in *E. coli* isolates from both humans and chickens strongly imply relationship between them. Such demonstration of high frequency of genetic profiles in ExPEC-associated virulence genes between ESBL-positive human and chicken isolates could mean their genetic relatedness. This definitely needs further molecular studies such as finger printing analysis to draw a conclusive statement.

A major limitation of this study was our inability to use any fingerprinting methods such as pulsed field gel electrophoresis (PFGE) or multi locus sequence typing (MLST) to delineate the genetic relatedness of the *E. coli* isolates from humans and the chickens.

5. Conclusion

Despite the limitation in the design of this analysis, the findings of this study revealed that the *TEM* gene was the predominant ESBL in chickens and the *CTX-M*-1 group was most prevalent in isolates from humans. There was no carbapenem and aminoglycosides resistance in the *E. coli* isolates from both humans and chickens. Analysis of the ExPEC-associated virulence profiles clearly demonstrates high occurrence rate in isolates from humans and chickens suggesting a probability of similarity in both cohort groups. Further studies are still needed to delineate this trend.

Acknowledgements

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