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Intestinal Tract of Broiler Chickens as a Reservoir of Potentially Pathogenic Curli Producing ESβL *Escherichia coli*

Ismail Raheel[1], Ahmed Orabi[2*], Ahmed Erfan[3], Mohamed Ashery Raslan[4], Shaimaa Hassan Abd El Wahab[5] and Eman A. A. Mohamed[6]

[1]Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Beni-Suief University, Egypt
[2]Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt
[3]Head of Biotechnology Unit, Animal Health Research Institute, Agriculture Research Center, Egypt
[4]Veterinarian; [5]Department of Microbiology, Animal Health Research Institute, Agriculture research center, Egypt
[6]Department of Microbiology, Faculty of Veterinary Medicine, Zagazig University, Egypt.
***Corresponding author:** drorabi2012@yahoo.com

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ABSTRACT

Commensal enteric *E. coli* in healthy birds have virulence genes which enable them to be pathogenic under suitable conditions. A total of 80 *E. coli* isolates recovered from the intestine of healthy broiler chickens were tested for some virulence characters based on Congo red (CR) binding assay, hemolysis, serum resistance and biofilm formation. The virulent isolates were identified serologically by slide agglutination test and O1 (25%) was the most prevalent enteric *E. coli* serogroups. The antibiogram profile revealed that all isolates were resistant to at least three different antibiotics. Amoxicillin and ceftriaxone showed the highest resistance (100%), followed by lincomycin, kanamycin, streptomycin and sulfamethoxazole-trimethoprim (96%). In contrast, 80 and 52% of the isolates were sensitive to amikacin and fosfomycin, respectively. The PCR revealed that all isolates harbored virulence genes (*int1, ampC, fimH, iss* and *eaeA*), in addition to 91, 16 and 16% of isolates had *mcr1, pic* and *tsh* genes, respectively, proving that avian intestinal reservoir of potentially pathogenic curli producing ESβL *Escherichia coli*.

Key words: Intestinal *E. coli,* Biofilm formation, Antimicrobial resistance, Virulence factors, Broiler chickens

INTRODUCTION

Escherichia coli are normal enteric inhabitant; however, it can be pathogenic that colonize the intestinal mucosa and cause diarrhea and extra intestinal diseases (Croxen et al. 2013; Tahir et al. 2021). Poultry intestine is the main reservoir of *E. coli* (Rodriguez-Siek et al. 2005) and there is continuous excretion (Ewers et al. 2005), which can cause serious infection in birds (McPeake et al. 2005). Potentially pathogenic *E. coli* can be detected by different in vitro phenotypic assays of Congo red binding which associated with presence of virulence genes such as *ompA, iss, crl* and *fimH*, and genes for multiple resistances to antibiotics (Zahid et al. 2016). In addition, in order to survive, the *E. coli* forms biofilm to evade the immune clearance and results in multiple antibiotics resistance (Hoffman et al. 2005). Detection of some *E. coli* serogroups as O1, O2, O8, O18 and O78, is a common method for determining pathogenic strains (Ewers et al. 2004). Development of resistance in *E. coli* is a world-wide public health problem, especially for 3rd generation cephalosporins due to extended spectrum beta-lactamases (ESβL) production (Machado et al. 2008) that results from high selective pressure on avian gut coliforms from antibiotic lavish use (Kabiswa et al. 2018) and caused by horizontal gene transfer (Vinué et al. 2006), acquisition of drug resistant genetic elements called integrons (de la Torre et al. 2014), or conversion of virulence genes in enteric *E. coli* into potential APEC "pAPEC" that can cause systemic disease (Ewers et al. 2009) and mediated by transferable genes from pathogenic to non-pathogenic strains and vice versa, in a multi-cultural environment like gut (Ogura et al. 2009), including fimbriae for colonization, *Iss* for serum survival, capsular and somatic antigens for anti-phagocytic activity, and a haemagglutinin sensitive to temperature *tsh* (Dziva and Stevens, 2008). Therefore, this study investigated a correlation between virulence capability and in vitro antibiotic resistance of *E. coli* isolated from intestine of healthy broilers.

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MATERIALS AND METHODS

Bio-Ethics Approval

This investigation was performed in accordance with the recommendations in the updated Guide for the Care and Use of Animals. All procedures were approved by the Institutional Animal Care and Use Committee Beni-Suef University, approval number 021-168.

Sampling and *E. coli* Isolation

A total of 150 samples collected from intestine of 2-5 weeks old healthy broiler chickens was inoculated into MacConkey's broth and incubated aerobically at 37°C for 24h and then subcultured onto MacConkey's agar, from which pink colonies (lactose fermenters) were subcultured onto EMB agar and incubated at 37°C for 24h. Suspected *E. coli* colonies with typical morphology were biochemically identified using API 20E kits "BioMe´rieux" (Koneman et al. 2001).

Phenotypic In-vitro Pathogenicity Tests

Congo Red Binding Assay

The CR-producing isolates were deep brick red colonies after incubation at 37C˚ for 24h on agar plates with 0.003% CR dye and 0.15% bile salt (Saha et al. 2020).

Assessment of Curli Production on YESCA CR Agar

E. coli strains were cultured onto LB plates and incubated at 37°C overnight. A single colony was streaked on a YESCA CR agar and incubated at 26°C for 48h. Most *E. coli* strains expressed curli in low salt and at low temperature were red, dark red or even black, while curli defective isolates were pink or white (Zhou et al. 2013).

Hemolysis Assay

Isolates were cultivated on sheep blood agar and incubated at 37°C for 24h. Hemolytic colonies produced clear zones of hemolysis (Vidotto et al. 1990).

Serum Resistance Assay

A 0.05mL of cell suspension ($2.5x10^8$ cfu/mL) in HBSS added to the same amount of serum were incubated at 37°C, then 10µL were plated on MHA and incubated at 37ºC. Serum bactericidal activity was expressed as percent of surviving bacteria after 180min and overnight incubation in relation to the original bacterial growth at 0min in control (Fecteau et al. 2001).

Serological Typing of *E. coli*

Virulent *E. coli* isolates were serologically identified using slide agglutination test using standard *E. coli* antisera (Edward and Ewing 1972).

Antimicrobial Susceptibility Test

The susceptibility of virulent isolates (1.5×10^8 CFU/mL) were tested using disc diffusion methods for 15 antibiotics (Oxoid), including kanamycin 30µg, streptomycin 10µg, amikacin 30µg, cefotaxime 30µg, ceftriaxone 30µg, ceftazidime 30µg, aztreonam 10µg, Doxycycline 30µg, colistin sulphate 10µg, lincomycin 10µg, amoxicillin 10µg, amoxicillin-clavulanic acid 20/10µg, ciprofloxacin 5µg, fosfomycin 50µg, and sulfamethoxazole trimethoprim 25µg (CLSI 2018).

Molecular Detection of Virulence and Resistance Genes

Twelve virulent MDR *E. coli* isolates based on in vitro virulence and antimicrobial resistance were tested by PCR (Bonnet et al. 2009) for the prevalence of 8 ExPEC virulence and resistance genes, including *int1* (Kashif et al. 2013), *fimH* (Ghanbarpour and Salehi 2010), *iss* (Yaguchi et al. 2007), *tsh* (Delicato et al. 2003), *eaeA* (Bisi-Johnson et al. 2011), *pic* (Boisen et al. 2009), *ampC* (Srinivasan et al. 2005) and *mcr1* (Newton-Foot et al. 2017).

RESULTS

A total of 80/150 (53%) *E. coli* isolates were recovered from intestine, of which 52 / 80 (65%) were CR positive and 35/ 80 (43.7%) were moderate or strong biofilm on a YESCA CR agar. The most prevelant serogroups of 28 phenotypically virulent *E. coli* isolates were O_1 (25%), followed by O_{128} and O_{119} (17.8% for each), then O_{86} and O_{158} (14.2% for each), while 3 (10.7%) were non-typed isolates. Antibiogram profile indicated that *E. coli* isolates were sensitive only to amikacin (80%) and fosfomycin (52%). All isolates were MDR and the highest resistance was to amoxicillin and ceftriaxone (100% for each) followed by lincomycin, kanamycin, streptomycin and SXT (96% for each), ciprofloxacin and doxycycline (88% for each), colistin sulphate (80%), aztreonam (72%), ceftazidime and amoxicillin-clavulanic acid (68% for each) and cefotaxime (64%). Occurrence of virulence and resistance genes was revealed by PCR as all isolates harboured *Int1, ampC, fimH, iss* and *eaeA* (100%), *Mcr1* (8/12, 66.7%) and *pic* and *tsh* genes (2/12, 16.7% for each) (Table 1).

DISCUSSION

Escherichia coli are ubiquitous bacteria adapted to different ecological niches either intestinal or extra intestinal sites and outside the host (Jang et al. 2017; Abdel et al. 2020; Hidayat et al. 2021). About 10-15% of pathogenic O serogroups possess virulence genes, acting as APEC reservoir that permit extra intestinal infection in poultry either through respiratory or digestive tract (Mellata et al. 2001). Enteric *E. coli* produce extracellular curli, a protease resistant cell surface proteinaceous amyloid fibers that bind to CR and other amyloid dyes (Zogaj et al. 2003), mediate surface attachment, cell–cell and host-pathogen interactions (Kikuchi et al. 2005) and increase bacterial resistance to antibiotics and stressors (Uhlich et al. 2006). In this study, 65% of *E. coli* isolates from intestine of healthy broilers were CR-positive that were considered as phenotypic pathogenicity marker (Zahid et al. 2016, Reichhardt and Cegelski 2018). This result matched with Berkhoff and Vinal (1986), who reported a correlation between CR expression and *E. coli* virulence. Biofilms are structured bacterial communities enclosed in a self- produced exo-polysaccharide matrix and adherent to abiotic or biological surfaces. Curli also play an important function in biofilm formation (Weiss-Muszkat et al. 2010). The results revealed that 43.7% of intestinal avian *E. coli* strains were able to form biofilm, while Skyberg et al. (2006) found that 75.7% of intestinal *E. coli* isolates from healthy broilers were able to form biofilm. Serotyping has been used as a method for identifying intestinal pathogenic *E. coli* "IPEC", but it does not discriminate APEC and AFEC (Ewers et al. 2009).

Table 1: Collective interrelation between virulence and resistance patterns of 12 MDR virulent intestinal *E. coli* serotypes

n=12 MDR virulent intestinal *E. coli* from broilers							
Serotypes	CRB ability	Biofilm formation	Hemolysis	Serum resistance (%)	Antimicrobial resistance pattern	resistance genes	Virulence genes
O1:k61	Positive	Positive	Positive	92	AML, CRO, k, S, SXT, MY, CIP, CT, ATM, AMC, FF	*Int*1, *amp*C	*fim*H, *iss*, *pic*, *eae*A
O1:k61	Positive	Positive	Positive	92	AML, CRO, k, S, SXT, MY, CIP, DO, CT, CTX, CAZ, AMC.	*Int*1, *Mcr*1, *amp*C	*fim*H, *iss*, *pic*, *eae*A
O1:k61	Positive	Positive	Positive	92	AML, CRO, k, S, MY, CIP, DO, CT, ATM, CTX, CAZ, AMC, FF, AK.	*Int*1, *Mcr*1, *amp*C	*fimH*, *iss*, *eaeA*
O1:k61	Positive	Positive	Positive	92	AML, CRO, k, S, SXT, MY, CIP, DO, CT, CTX, CAZ, FF.	*Int*1, *Mcr*1, *amp*C	*fimH*, *iss*, *eaeA*
O128:K71	Positive	Positive	Positive	94	AML, CRO, k, S, SXT, MY, CIP, DO, CT, ATM, CAZ, FF.	*Int*1, *Mcr*1, *amp*C	*fim*H, *iss*, *eae*A
O128:K71	Positive	Positive	Positive	94	AML, CRO, k, S, SXT, MY, CIP, CT, ATM, AMC, FF.	*Int*1, *Mcr*1, *amp*C	*fimH*, *iss*, *eaeA*
O119:K58	Positive	Positive	Positive	88	AML, CRO, k, S, SXT, MY, DO, CT, ATM, CTX, CAZ, AMC.	*Int*1, *Mcr*1, *amp*C	*fim*H, *iss*, *eae*A
O119:K69	Positive	Positive	Positive	90	AML, CRO, k, S, SXT, CIP, DO, CT, ATM, CTX, CAZ, AMC, AK.	*Int*1, *Mcr*1, *amp*C	*fim*H, *iss*, *eae*A
O86:k61	Positive	Positive	Positive	85	AML, CRO, k, S, SXT, MY, CIP, DO, ATM, CTX, CAZ, FF, AK.	*Int*1, *Mcr*1, *amp*C	*fim*H, *iss*, *tsh*, *eae*A
O86:K61	Positive	Positive	Positive	85	AML, CRO, k, S, SXT, MY, CIP, DO, CT, ATM, CAZ.	*Int*1, *Mcr*1, *amp*C	*fim*H, *iss*, *tsh*, *eae*A
O158:k-	Positive	Positive	Positive	95	AML, CRO, SXT, MY, CT, ATM, CTX, AMC.	*Int*1, *Mcr*1, *amp*C	*fim*H, *iss*, *eae*A
O158:K-	Positive	Positive	Positive	95	AML, CRO, k, S, SXT, MY, CIP, DO, ATM, CTX, AMC, FF, AK.	*Int*1, *Mcr*1, *amp*C	*fim*H, *iss*, *eae*A

MDR: multi drug resistant, CRB: Congo red binding, AML: amoxicillin 10µg, CRO: ceftriaxone 30µg, k: kanamycin 30µg, S: streptomycin 10µg, SXT: sulfamethoxazole trimethoprim 25µg, MY: lincomycin 10µg, CIP: ciprofloxacin 5µg, DO: Doxycycline 30µg, CT: colistin sulphate 10µg, ATM: aztreonam 10µg, CTX: cefotaxime 30µg, CAZ: ceftazidime 30µg, AMC: amoxicillin-clavulanic acid 20/10µg, FF: fosfomycin 50µg, AK; amikacin 30µg, *int*1: integrase genes, *Mcr*1: plasmid-mediated resistance to colistin, *amp*C; ampicillinase C plasmid-mediated inducible β-lactamase, *fim*H: fimbrin D-mannose specific adhesin , *iss:* increased serum survival, *pic:* Serine protease pic autotransporter, Involved in intestinal colonization, *tsh:* Temperature-sensitive haemagglutinin *tsh* autotransporter, *eae*A: attaching and effacing intimin gene A.

In the current study, the most prevelant serogroups were O_1 (25%) followed by O_{128} and O_{119} (17.8% for each) and O_{86} and O_{158} (14.2% for each). Unfourtunitly, significant percentage of isolates (10.7%) were untyped, whereas O_2 and O_{78} serotypes were not detected in this study although they are the most common APEC serogroups (McPeake et al. 2005; Yaguchi et al. 2007). In poultry industery, evolution of multi drug resistant *E. coli* strains and resistance genes transmission is a problem in APEC infection control (Subedi et al. 2018), owing to antimicrobial misuse which develops commensal resistant bacteria acting as reservoir of resistance genes for pathogenic species (Founou et al. 2016). Biofilm formation is considered virulence criteria of pathogenic *E. coli* strains, leading to persistence infections resulted from treatment failure (Römling and Balsalobre 2012).

The current study showed that all 25 tested biofilm forming *E. coli* isolates were MDR that were resistant to 3 or more antibiotic classes and this is in accordance with Dube and Mbanga (2018). Complete resistance were observed for amoxicillin and ceftriaxone (100%), followed by lincomycin, kanamycin, streptomycin and sulfamethoxazole trimethoprim (96% for each), ciprofloxacin and doxycycline (88% for each) and colistin sulphate (80%). However Subedi et al. (2018) found that 94% of *E. coli* isolates from broiler chickens were MDR with highest resistance to ampicillin (98%), followed by doxycycline (62%). After phenotypic screening, 3 antimicrobial resistance genes (*int1*, *ampC* and *mcr1*) were detected in 12 MDR *E. coli* isolates using PCR as 100%, 100% and 91%, respectively. Mobile genetic elements as plasmids, transposons and integrons carrying resistance genes facilitate their rapid transfer among bacteria (Sunde and Norström 2006). Integrons are mobile genetic elements able to capture, integrate and express antimicrobial resistance gene cassettes (Mazel 2006). Extended-spectrum β-lactamase which is plasmid mediated is capable of hydrolyzing β-lactam antibiotics leading to bacterial resistance (Ceccarelli et al. 2019). Colistin resistance is the last resort antibiotics for treatment of MDR *Enterobacteriaceae*. Nine *mcr* genes (1-9) are mobile, since they are plasmid mediated (Hoelzer et al. 2017; Zhang et al. 2018; Ahmed et al. 2020).

In our study, transferable colistin resistance gene *mcr-1* was identified in 91% of isolates and this explain the high un expectable resistance of *E. coli* to Colistin sulphate (80%). APEC virulence-associated properties include adhesion to respiratory and digestive tracts (Moulin and Fairbrother 1999). The main adhesins identified in APEC were *FimH* family, one of type 1 fimbrial adhesins that agglutinate fowl erythrocytes, curli fibers, which are thin aggregative surface fibers, temperature-sensitive hemagglutinin *"tsh",* which is a member of auto transporter proteins helping in lesion formation and fibrin deposition in avian air sacs (Dozois et al. 2000), *eaeA*, a gene necessary for attachment of entero-pathogenic *E. coli* to epithelial cells (Donnenberg et al. 1993), *Iss* associated with complement resistance (Moulin and Fairbrother

1999), a Serine protease *pic* gene auto transporter, involved in *E. coli* intestinal colonization, serum resistance, heme agglutination and intestinal mucus layer penetration (Henderson et al. 1999). In the present study, PCR showed that all the 12 tested isolates harbored *fimH*, *iss* and *eaeA* (100%), *pic* and *tsh* genes (2/12, 16%). However, Delicato et al. (2003) detected *fimH*, *iss* and *tsh* genes in 92%, 8% and 4% of *E. coli* isolates from healthy chickens, respectively. In conclusion, gut *E. coli* of broiler chickens have a vital role in colonization and pathogenicity spread as they carry antibiotic resistance genes in mobile genetic elements that can transmit resistances. Therefore, it is crucial to develop precautionary measures that control infection in poultry and food contamination.

Author's Contribution

All authors contributed equally to study the design methodology, interpretation of results, and writing of the manuscript.

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