SHORT COMMUNICATION

Veterinary Research Forum. 2021; 12 (4) 505 – 510
doi: 10.30466/vrf.2020.115921.2762
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Molecular detection and analysis of virulence genes in multi-drug resistant *Escherichia coli* from infected broilers

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Article Info

Article history:

Received: 20 October 2019
Accepted: 01 June 2020
Available online: 15 December 2021

Keywords:

16s rRNA sequencing
Commercial broilers
Molecular detection
Multi-drug resistant *E. coli*
Virulence genes

Abstract

Escherichia coli associated infections are major threats in poultry industry owing to severe economic losses each year. This study was conducted to identify *E. coli* isolates, to evaluate their antibiotic sensitivity and to find out their virulence patterns from infected broilers of Sylhet city in Bangladesh. Using polymerase chain reaction, a total 20 isolates were identified as *E. coli* from 11 chickens, exhibiting symptoms like colibacillosis and/or diarrhea. All isolates were positive for type-1 fimbrial adhesion (*fimH*), followed by putative avian hemolysin (*hlyF*) in 17 isolates; while none of the isolates was amplified with intimin (*eaeA*). Among 10 tested antibiotics, 100% of the isolates (n = 20) showed resistance to ampicillin, amoxicillin and tetracycline; but they were 100% sensitive to gentamicin. Organ specific correlations of antibiotic sensitivity were obtained among the isolates through principal component analysis (PCA) and Agglomerative Hierarchical Clustering (AHC). The 16S rRNA data of two multi-drug resistant isolates revealed closed clustering with clinical *E. coli* strains which could be indication of their zoonotic potential. In conclusion, the results depict higher prevalence of *fimH* and *hlyF* genes and drug resistance patterns of *E. coli* isolates from broilers in Sylhet city of Bangladesh.

Introduction

Poultry sector plays a substantial role in fostering worldwide agricultural growth and reducing malnutrition in developing country like Bangladesh. It contributes about 22.00 - 27.00% of total animal protein supply in the country and has created job opportunity for more than six million people.[1] However, owing to prevalence of diseases, the performance of poultry production is greatly impaired and *Escherichia coli* has been regarded as a prominent pathogen towards this effect. Bacterial diseases such as colibacillosis caused by avian pathogenic *E. coli* reflect heavy economic losses in broilers and layers due to morbidity and mortality.[2] Although several biochemical and molecular techniques are being used for diagnosis, polymerase chain reaction (PCR) and 16S rRNA based sequencing have been proved to be very promising in detection of bacterial species due to their sensitivity and accuracy.[3]

Antibiotic therapy is one of the primary control measures for reducing the *E. coli* associated infections in poultry; though, drug resistance has become a growing concern over the last couple of decades.[4] It also makes the treatment process expensive, complicated and time-consuming. Several virulence factors have been associated with the pathogenesis of *E. coli* including type 1 fimbrial adhesion (*fimH*), putative avian hemolysin (*hlyF*) and intimin (*eaeA*).[5] Type 1 fimbrial adhesion protein stabilizes the adhesion to host urinary epithelium under shear stress; while, hemolysin lyses erythrocytes by forming pores or hydrolyzing the phospholipids in phospholipid bilayers.[6] Intimin is expressed on the bacterial cell surface and mediates host cell interaction through attaching and effacing lesions.[7]

Although, poultry industry plays a significant role in supplying national demand of protein sources, the information regarding pathogenesis and control of *E. coli* associated poultry diseases is still limited. Apart from

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phenotypic characterization, precise identification of virulence genes and anti-microbial resistance pattern of pathogenic *E. coli* are yet to be extensively studied in Bangladesh. Some works have been carried out regarding the risk of anti-microbial resistance and virulence of *E. coli* from poultry and their feeds in other countries.[8,9] However, due to the bacterial diversity in different habitats/regions, region specific studies are needed to globally combat bacterial drug resistance. Hence, the study was designed to determine antibiogram profile and virulence gene of *E. coli* from infected broilers in Bangladesh. The possible risk of zoonosis on the basis of phylogenetic data and organ specific correlation of anti-microbial resistance were indicated in the study can render an important research area to be dealt in future.

Materials and Methods

Sample collection. A total of 11 broiler chickens (aged 2 - 4 weeks) manifested signs and symptoms of colibacillosis and/or diarrhea were collected from five different poultry farms (commercial farms with population range of 500 - 1000) of Sylhet City in Bangladesh and transported immediately to the Laboratory of Microbial Biotechnology at Sylhet Agricultural University, Sylhet, Bangladesh.

Isolation. Pieces of liver, heart, lungs, gizzard, small intestine tissues and cloacal swab were collected and inoculated in 55 separate nutrient broth (HiMedia, Mumbai, India) using sterilized cotton for 24 hr at 37.00 ˚C. Samples were then sub-cultured on Eosin methylene blue (EMB) agar (HiMedia) for overnight at 37.00 ˚C. The presumptive identification of *E. coli* based on phenotypic characteristics was performed using methods described earlier.[8]

DNA extraction. A total of 20 bacterial isolates were inoculated in nutrient broth and incubated overnight at 37.00 ˚C. The genomic DNA was extracted using commercial FavorPrep Tissue Genomic DNA Extraction Mini kit (Favorgen Biotech Corp., Ping-Tung, Taiwan) following manufacturer's instructions. The extracted DNA was checked for quality in 0.80% agarose gel followed by visualization under UVP Benchtop UV Transilluminator (Thermo Fisher Scientific, Waltham, USA) and quantified using NanoDrop™ Spectro-photometer (Thermo Fisher Scientific).

Molecular identification. A pair of gene specific primer, eco-F (5′-GACCTCGGTTTAGTTCACAGA-3′) and eco-R (5′-CACACGCTGACGCTGACCA-3′), was used to amplify the ecotin precursor gene (*eco*) through PCR to identify *E. coli* at molecular level.[10] Master mix was prepared at a volume of 25.00 µL of reaction mixture containing 3.00 µL template DNA, 12.50 µL GoTaq G2 green master mix (Promega Corporation, Madison, USA), 1.50 µL of each forward and reverse primers (Integrated DNA Technologies, Inc., San Diego, USA) and 6.50 µL of nuclease-free water. Thirty-five cycles of amplification reactions were carried out in a MultiGene gradient thermal cycler (Labnet International Inc., Edison, USA) involving initial denaturation step at 94.00 ˚C for 3 min, denaturation step at 94.00 ˚C for 1 min, annealing 56.00 ˚C for 1 min and a final extension at 72.00 ˚C for 2 min with 35 serial cycles of reactions. The amplified PCR products were separated using 1.00% agarose gel and visualization by gel documentation system.

Antibiotic sensitivity test. Antibiogram assay of the *E. coli* isolates was performed according to the guidelines of Clinical and Laboratory Standard Institute (CLSI) following disc diffusion method using Mueller Hinton Agar (HiMedia).[8] A total of 10 commercial antibiotics (HiMedia) were used to evaluate the sensitivity pattern. The inhibition zones produced around the antibiotic discs were measured according to CLSI method[11] and categorized as a resistance, intermediate resistance or susceptible.

Molecular identification of virulent genes. Three pairs of gene specific primer, *fimH*-F (5′-GGATAAGCCG TGGCCGGTGG-3′) and *fimH*-R (5′-CTGCGGTTGTGCCG GAGAGG-3′), *hlyF*-F (5′-GGCCACAGTCGTTTAGGGTGC TTACC-3′) and *hlyF*-R (5′-GGCGGTTTAGGCATTCCGA TACTCAG-3′) and *eaeA*-F (5′-TGCGGCACAACAGGCGG CGA-3′) and *eaeA*-R (5′-CGGTCGCCGCACC AGGATTC-3′) from the Integrated DNA Technologies Inc., were used to detect *fimH*, *hlyF* and *eaeA* genes, respectively.[12,13,19] The PCR reaction was optimized with an initial denaturation step at 94.00 ˚C for 3 min, denaturation step at 94.00 ˚C for 1 min, an annealing (58.00 ˚C, 57.00 ˚C and 61.00 ˚C for *fimH, hlyF* and *eaeA*, respectively) and a final extension at 72.00 ˚C for 2 min with 35 serial cycles of reactions.

Sequencing and analysis of multi-drug resistant *E. coli* isolates. Two isolates named I1 and GB2 showing resistance to at least three antibiotics containing both *fimH* and *hlyF* genes were selected for 16S rRNA gene sequencing. The DNA samples were amplified using 27F and 1492R universal sequencing primers as described.[14] The amplified PCR products were sent to DNA Solutions Ltd. (Dhaka, Bangladesh), by maintaining cool chain (4.00 ˚C) for Sanger sequencing. Extracted raw sequences were checked for quality in Chromas (version 2.01, Technelysium Pty Ltd., South Brisbane, Australia) and further processed in Bioedit (version 7.0.4; North Carolina State University, Raleigh, USA). The DECIPHER (version 9.20; Sanger Institute, Cambridgeshire, UK) was used to remove chimeras from the sequences following assembly in SeqMan Pro (version 15.0; DNASTAR Inc., Madison, USA). Highly similar sequences (> 97.00%), were obtained through BLASTn search with default parameters.

Multiple sequence alignment was performed using ClustalW in MEGA Software (version 7.0; Biodesign Institute, Tempe, USA) followed by phylogenetic tree construction using neighbour-joining Kimura-J model with 1,000 bootstrap replicates.

Statistical analysis. The results of this study were analyzed using SPSS Software (version 23.0; IBM Corp., Armonk, USA). One-way analysis of variance (ANOVA) was used to calculate any significant differences ($p < 0.05$) among the sensitivity patterns of different antibiotics against *E. coli* isolates. The principal component analysis (PCA) and Agglomerative Hierarchical Clustering (AHC) were performed in XLSTAT to analyze the correlations among different variables of diseased boilers including numbers, sources, age and organs with antibiotic resistant patterns.

Results

Molecular identification of *E. coli* isolates. A total of 20 isolates out of 55 cultures showed growth in EMB agar and characteristic greenish metallic shine. Identification of these 20 isolates was further confirmed as *E. coli* through PCR. Primer specific bands for *eco* gene (585 bp amplicons) were identified by 1.00% agarose gel electrophoresis. The 16S rRNA characterization followed by subsequent phylogenetic tree construction of two multi-drug resistant *E. coli* revealed a close positioning of poultry isolates with some clinical isolates, recently characterized from Sylhet city, Bangladesh (Fig. 1A). The study sequences are currently available at National Centre for Biotechnology Information databank with the accession of MK719862 and MK719863 for isolate I1 and GB2, respectively.

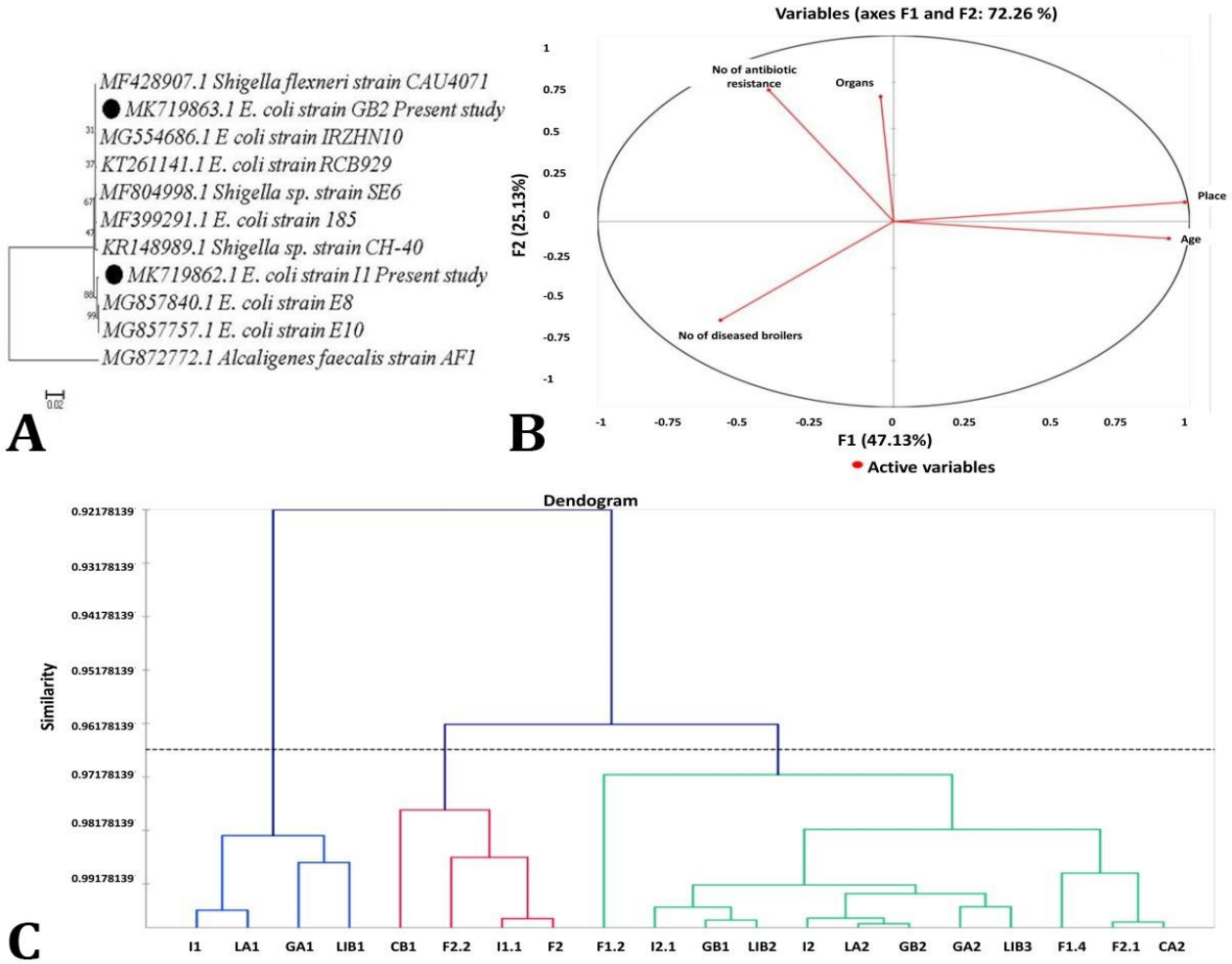

Fig. 1. A) Neighbor-joining phylogenetic tree showing the relationship of studied *E. coli* isolates with bacteria characterized from clinical samples. Isolate I1 positioned very close to *E. coli* strain E8 and E10 (two most recent circulating isolates in the study area); while isolate GB2 found to have significant sequence detachment from I1 and clustered with other global circulating *E. coli* and *Shigella sp.* isolates. **B)** Principal component analysis (PCA) plot showing the correlations among variables (together axes F1 and F2 describe 72.26% variables; where, antibiotic resistance was found to have positive correlation with organs). **C)** Agglomerative Hierarchical Clustering (AHC) study of *E. coli* isolates on the basis of five variables (organs, age, place, number of diseased boiler and antibiotic resistance). The first group (displayed in green color) is more homogenous than the other two groups.

Antibiogram profiling. The *E. coli* isolates were found to be variable in their antibiotic sensitivity pattern against 10 anti-microbial agents (Table 1). All the isolates were resistant to at least three antibiotics and determined as multi-drug resistant. The isolates were 100% resistant to ampicillin (10.00 µg per disc), amoxicillin (30.00 µg per disc) and tetracycline (30.00 µg per disc). Noticeable resistance pattern was observed against vancomycin (30.00 µg per disc) and neomycin (30.00 µg per disc) by 95.00% and 85.00%, respectively (Table 1). However, all isolates were 100% sensitive to gentamicin (10.00 µg per disc). The PCA plot described 72.26% variables; where, antibiotic resistance found to have positive correlation with organs (Fig. 1B). The AHC also showed organ-specific positive correlation which was more homogenous than other two groups (Fig. 1C).

PCR-based identification of virulent genes. The *fimH* gene was present among all *E. coli* isolates (20) with expected PCR product of 331 bp (Fig. 2). However, the distributions of other genes in the isolates were found to be variable. The *hlyF* gene was amplified and detected in 17 isolates (except I1.1, LIB2 and I2) with expected amplicons of 450 bp (Fig. 2); while no isolates showed positive result for *eaeA* gene.

Table 1. Antibiogram profile of *E. coli* isolates and sensitivity pattern to different antibiotics. The number given after the name of each agent is the dosage per disc in micrograms.

Isolates	Gentamicin (10.00)	Neomycin (30.00)	Vancomycin (30.00)	Tetracycline (30.00)	Ampicillin (10.00)	Streptomycin (10.00)	Amoxicillin (30.00)	Kanamycin (30.00)	Ciprofloxacin (5.00)	Norfloxacin (10.00)
I1	++++	+++	++	++	++	++++	++	++	+++	++
F1.2	++++	++++	++	++	++	++++	++	+++	++++	++++
GA1	++++	++	++	++	++	+++	++	++	++	++
LA1	++++	+++	++	++	++	++++	++	++	++	++
LIB1	++++	+++	++	++	++	++	++	++	++	++
F1.4	++++	++++	++	++	++	++++	++	+++	+++	++++
CB1	++++	++	++	++	++	++	++	++++	++++	++++
I1.1	++++	++++	++	++	++	++	++	++	++++	++++
GB1	++++	+++	++	++	++	++++	++	++++	++++	++++
F2	++++	++	++	++	++	++++	++	++	++	++
F2.1	++++	+++	+++	++	++	+++	++	+++	++++	++++
LIB2	++++	+++	++	++	++	++	++	+++	++++	++++
LA2	++++	+++	++	++	++	+++	++	+++	++++	++++
CA2	++++	+++	++	++	++	++++	++	++	++++	++++
I2	++++	+++	+++	++	++	++++	++	+++	++++	++++
GB2	++++	+++	++	++	++	++++	++	++++	++++	++++
GA2	++++	+++	++	++	++	+++	++	+++	++	+++
I2.1	++++	+++	++	++	++	++++	++	++++	++	++
F2.2	++++	+++	++	++	++	++	++	++	++	++
LIB3	++++	+++	+++	++	++	++++	++	++++	+++	+++

* Resistant (++), Intermediate resistant (+++), and Susceptible (++++).

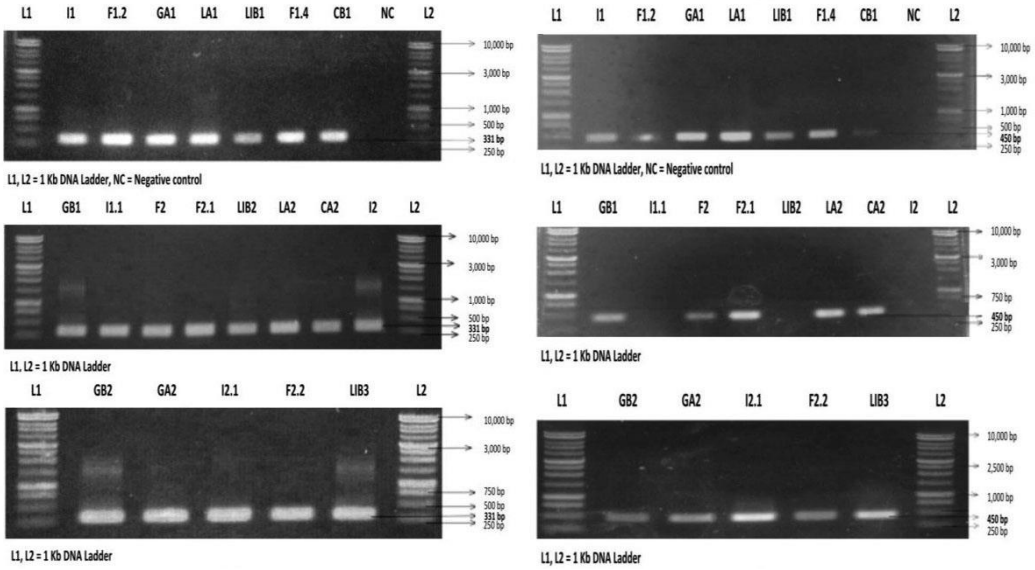

Fig. 2. Polymerase chain reaction detection of type 1 fimbrial adhesion (*fimH)* and hemolysin (*hly)* genes (331 bp and 450 bp amplicons confirmed the presence of *fimH* and *hly* among the *E. coli* isolates, respectively).

Discussion

Determining virulence genes and profiling antibiogram in bacteria have been regarded as far-reaching works to know the pathogenesis and potential risks of anti-microbial resistance. In the present study, the phylogenetic analysis of two multi-drug resistant *E. coli* isolates, I1 (MK719862) and GB2 (MK719863) revealed a close positioning of the *E. coli* isolates with some clinical isolates,[14] recently characterized from Sylhet city, Bangladesh. The phylogenetic analysis also indicates that the isolates might have zoonotic potential to transmit human and cause diseases.[15,16] While, in most of the cases, the poultry workers are always in contact with chickens without any protective measures in Bangladesh; henceforth, there could be a high risk of zoonosis deserving future attention.

Emergence of multi-drug resistant *E. coli* is substantially increasing due to the abuse of antibiotics. Here, sensitivity of all isolates to gentamicin indicates the potential efficacy of this antibiotic against *E. coli*. Our study found organ-specific sensitivity patterns of antibiotics for *E. coli* isolates in correlation analysis; where, the isolates from cloaca (F2 and F2.2) and intestine (LIB1) showed more resistance than others (resistant to 8 antibiotics). Previous study has reported internal organ specific antibiotic response in different microbes as well.[17] Consistent with previous study, we also observed similar trend of sensitivity. Hence, we believe a distinctive pattern of antibiotic resistance can be obtained depending on diversity of isolates source. This could be a novel and impressive area of anti-microbial research where the researchers should come forward. However, to compare the antibiogram results for other organs, there are not enough data currently available to compare with; thus, further investigation with more samples is necessary.

Identification of virulence factors is the key for understanding bacterial pathogenesis and interactions within the host.[18] Here, the presence of *fimH* in all isolates exhibited better similarity with previous work of Van der Westhuizen and Bragg and Rodriguez-Siek *et al.*, by 100% and 98.10 %, respectively.[19,20] The study also detected the presence of *hlyF* in 17 of the 20 isolates, excluding I1.1, LIB2 and I2. However, *eaeA* gene was observed among the isolates. Similar result was obtained by Kiliç *et al.*, reporting 10 of the 401 *E. coli* strains from chickens possessed *eaeA* gene.[21] It could be assumed that the feeding sources, microflora of chicken gut, environment of the chicken farm and genetic lineage of the broiler might influence the virulence genes distribution in these isolates.

In conclusion, the study depicts higher prevalence of *fimH* and *hlyF* genes and multi-drug resistant *E. coli* isolates from broilers in Sylhet city of Bangladesh. Our research could help to understand the virulence of *E. coli* induced diseases in poultry and to determine suitable control measures of those diseases. Moreover, detailed epidemiological studies are necessary to know the potential risk of this pathogen transmission to human.

Acknowledgments

The work was under the Research and Development Project (2016-17) titled "Molecular detection of virulence genes and *in vitro* pathogenesis of *E. coli* isolates causing diseases in broiler chicken" funded by the Ministry of Science and Technology, Bangladesh. The authors are also thankful to Sylhet Agricultural University Research System (SAURES) for guidance in conducting the project. Authors also express indebtedness to The Department of Genetic Engineering and Biotechnology of Shahjalal University of Science and Technology, Sylhet, Bangladesh, for their PCR facilities during the experiment.

Conflict of interest

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by the authors.

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