Plant Tissue Cult. & Biotech. **33**(1): 17-24, 2023 (June) **ISSN 1817-3721, E-ISSN 1818-8745**
DOI: https://doi.org/10.3329/ptcb.v33i1.66063

PTC&B

Validation of a Multiplex PCR for the Simultaneous Detection of *E. coli* and Class 1 Integron

Asif Md. Fuad Hasan, Anindita Bhowmik, Mohammad Nurul Islam[1] and Sunjukta Ahsan*

Department of Microbiology, University of Dhaka, Dhaka-1000, Bangladesh

Key words: E. coli, antibiotic resistance, Class 1 Integron, PCR

Abstract

Antibiotic resistant *Escherichia coli* in the animal gut can promote the transfer of antibiotic resistance between animal and human. In order to enforce the One Health National Action Plan in Bangladesh, it is imperative to detect the presence of *E. coli* harbouring Class 1 integrons that can potentially transfer antibiotic resistance genes among bacteria. Previously used primers pairs were used in a multiplex PCR to successfully detect the *E. coli* species-specific amplicon and Class 1 integron in 57% of the isolates. Plasmid was absent in the isolates. Antibiotic resistance profiling indicated highest resistance to oxytetracycline (89%) and lowest resistance to streptomycin (18.52%). A total of 38% isolates were found to be resistant against 8 of the 10 antibiotics used, 85.19% being multidrug resistant. However, the resistance pattern could not be specifically correlated to the presence of Class 1 integron. The validated multiplex PCR can be used as a quick method to detect *E. coli* and Class 1 integron simultaneously.

Introduction

Escherichia coli is the etiological agent of community-acquired and nosocomial infections in human (Briñas et al. 2002). It may be transmitted to human in a variety of ways, including food, water and animals. In addition to being a pathogen, *E. coli* may become resistant to a number of drugs. Drug resistance is a serious threat for human health as this may interfere with treatment regime. Integrons can carry multidrug resistance (MDR) genes among bacteria, including *E. coli* through horizontal gene transfer (HGT). The ability of bacteria to acquire antibiotic resistance genes by HGT has played a crucial role in the dissemination and development of MDR in bacteria (Leverstein et al. 2003). Integrons have the ability to acquire genes in the form of gene cassettes (Machado et al. 2005). There are five broad classes of integrons (Classes 1, 2, 3, 4 and 5) based on the

*Author for correspondence: <sunjukta@du.ac.bd>. [1]Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh.

presence of distinct integrase genes (Mazel 2006). Class 1 integrons are ubiquitously found in enteric bacteria including *E. coli* (Goldstein et al. 2001). They are more prevalent than other classes of integrons owing to their ability to recombine with different *attC* sites and to their flexibility in their specificity of nucleotide sequences (Bogaard et al. 2001). Integrons harbor a variety of gene cassettes, the most common of which confer resistance to aminoglycosides, streptomycin and spectinomycin, trimethoprim and erythromycin (White 2000, 2001). Integrons belonging to classes 1, 2 and 3 are present in members of the *Enterobacteriaceae* and Class 1 integrons are most predominant in enteric bacteria including *E. coli* (Goldstein et al. 2001). Class 1 integrons contain bacterial resistance to aminoglycosides and sulphonamides (Hall et al. 1995).

In Bangladesh, antibiotics are extensively used as growth promoters in poultry production or to control infectious disease. The use of antibiotics must be minimized in order to prevent the rise of antibiotic resistance (Moreno et al. 2009). Likewise, antibiotic usage can lead to the emergence and dissemination of resistant *E. coli* which can then be passed into people via food or direct contact with infected animals. These resistant microorganisms may function as a potential source in the transportation of antimicrobial resistance to human pathogens (Bogaard et al. 2001, Schroeder et al. 2002). The present study was aimed to validate the application of existing primer pairs for the simultaneous detection of *E. coli* and Class 1 integrons in fecal samples from animals. Rapid detection of *E. coli* containing Class 1 integron is an indication of the presence and possible transmissibility of antibiotic resistance among bacteria. Following detection, control measures to reduce drug transfer can be taken accordingly, thereby aiding the One Health National Action Plan (www.who.int).

Materials and Methods

Glycerol stock of *Escherichia coli* was prepared using pure culture of bacteria isolated from poultry feces. Samples were given identification numbers in the form of digits followed by numerals (e.g. A1, A13, A71 etc.). For the revival of bacterial samples from glycerol stocks, Tryptic Soy Broth (TSB) was used. Fifty (50) micro liter of the stock was transferred to 200 micro liter of TSB in an Eppendorf tube with a micropipette. The inoculated tubes were then incubated at 37°C for 24 hours.

A loop full of bacteria was transferred to MacConkey agar plates by streaking in a zig zag manner. The plates were incubated at 37°C for 24 hours. Thereafter, colony characteristics were noted. To perform antibiotic sensitivity test, isolates were inoculated in nutrient broth and incubated in shaker incubator at 37°C for 4 hours to obtain logarithmic phase culture. Bacterial lawn was prepared by dipping sterile cotton swabs in Mueller Hinton agar plates. A total of 10 antibiotic discs (oxytetracycline, azithromycin, streptomycin, amoxycillin. tetracycline, erythromycin, cefixime, co-trimoxazole, chloramphenicol and ciprofloxacin) were tested. All the plates were incubated overnight at 37°C. Zone diameters were measured and compared with CLSI

(2020) guidelines to determine antibiotic sensitivity profiles. From the MacConkey Agar plates, bacteria were streaked on Blood Agar and incubated at 37°C for 24 hours. The plates were observed for zones of haemolysis. The plasmid extraction kit from Vivant technologies (Malaysia) was used. The protocol for the extraction was provided in the kit. Following plasmid extraction, 10µl of purified sample was loaded in 1% agarose gel for resolution. 1kb ladder (GeneOn, Taiwan) was used for size determination.

PCR was used to specifically amplify the *intI* gene specific for Integron Class 1. To perform PCR, fresh culture of all the bacterial samples were prepared by subculturing in nutrient agar. Then colony PCR was carried out for each sample and for each target in separate PCR tubes. For colony PCR, a medium sized single colony was transferred with sterile toothpick for each bacterium and resuspended in nuclease free water. The suspension was heated to lyse the cells and release DNA. Lysed cell suspension was centrifuged for 3-5 minutes to precipitate cell debris. From the supernatant, 2µl was used as template DNA for PCR. The multiplex PCR reaction mixture contained 46 µl water, 50 µl master mix (2X), 1 µl DMSO, 1 µl $MgCl_2$ and 0.5 µl of each primer (*intI*-1 forward). 5′ACATGTGAGGCGACGCACGA-3′ , *intI*-1 reverse 5′-ATTTCTGTCCTG GCT GGCGA-3′ for Integron and *uidA* forward 5′-TATGGAATTTCGCCGATTTT -3′ and *uidA* reverse 5′-TGTTTGCCTCCCTGCTGCGG -3′ for species-specific PCR (Godambe et al. 2017, Goldstein et al. 2001).

The PCR program used 10 minutes of initial denaturation at 95°C followed by 35 cycles each consisting of denaturation at 95°C for 30 seconds, annealing at 55°C for 45 seconds and extension at 68°C for 60 seconds. A final extension was carried out at 68°C for 10 minutes. PCR products were resolved in agarose gel (1% in TAE buffer). A 1kb ladder (GeneOn, Taiwan) was used. Electrophoresis was carried out at 85V for 1 hour. After staining in ethidium bromide for 30 minutes, the gels were viewed in a transilluminator.

Results and Discussion

Escherichia coli, is a common inhabitant of the gastrointestinal tract of animals and humans as well. It is also an important human pathogen involved in intestinal and extra-intestinal infections. The use of multiple antibiotics in humans over a long period of time has contributed to the emergence of MDR *E. coli*. Several studies have indicated a strong association between antibiotic resistance and integrons, indicating the significance of integrons in the spread of such resistance determinants (Sabbagh et al. 2021).

In Bangladesh, antibiotics are used at sub-therapeutic levels to improve growth and production of cattle. The One Health concept stresses the ecological relationship between human, animal and the environment. Bangladesh is implementing the National Action Plan (NAP) for containing Antimicrobial Resistance (AMR) in human, animal, and environment sectors through “One Health” approach where the Department of Livestock Services (DLS) is the mandated body to implement NAP strategies in the animal health

sector of the country (Ahmed et al. 2022). The detection of antibiotic resistant *E. coli* in farm animals is therefore an important target area to implementing the NAP in Bangladesh.

As with antibiotic resistance, it is also imperative to detect Integrons, because these are commonly found in bacteria and are involved in antibiotic resistance and its transfer. Upon determination of antibiotic sensitivity, highest resistance was observed to oxytetracycline (88.89%) and lowest to streptomycin (18.52%). A total of 38% isolates were resistant to 8 of the 10 antibiotics used, 85.19% being multidrug resistant. Zone sizes were compared with MIC and zone distribution of CLSI (2020) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines. According to the guidelines sample bacteria were designated as resistant (R), intermediate (I) and sensitive (S) in Table 1 and Fig. 1.

Table 1. Antibiotic sensitivity pattern of the isolated bacteria.

Antibiotic	Resistant (%)	Intermediate (%)	Sensitive (%)
Streptomycin	18.52	74.07	7.41
Cefixime	70.37	29.63	0.00
Chloramphenicol	25.93	0.00	74.07
Ciprofloxacin	33.33	7.41	59.26
Azithromycin	40.74	0.00	59.26
Amoxicillin	59.26	0.00	40.74
Oxytetracycline	88.89	3.70	7.41
Co-Trimoxazole	59.26	0.00	40.74
Tetracycline	77.78	0.00	22.22
Erythromycin	88.89	0.00	11.11

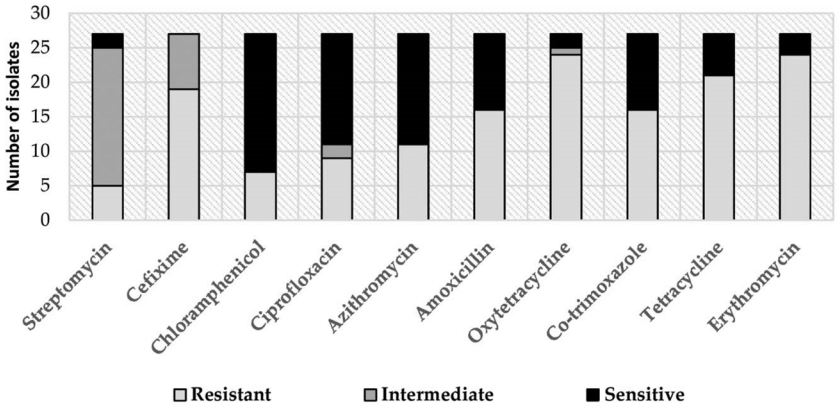

Fig. 1. Antibiotic sensitivity profile of the identified bacterial isolates

None of the isolates were found to contain plasmids. However, as the kit cannot purify megaplasmids, additional plasmid purification method needs to be carried out to determine the presence of large plasmids. Isolates with Integrons have a greater tendency to acquire resistance to antibiotics than those without integrons **(**Holger et al. 2011). Integrons are usually not mobile by themselves but are transported by transposons/plasmids (Sabbagh et al. 2021). Integron carries antibiotic resistance genes and are easily transferred with the aid of transposons (Cambray et al. 2010). Various classes of integrons have been widely reported in *E. coli* isolated from farm animals, including cattle poultry, and aquatic animals (Zhang et al. 2009, Cocchi et al. 2007) as well as in companion animals such as cats and dogs (Goldstein et al. 2001, Cochi et al. 2007). Farm animals fed antibiotics as growth promoters contain Class 1 integrons (Cocchi et al. 2007).

Multiplex PCR was carried out for the detection of Class 1 integron and *uidA* (*E. coli* species specific gene) fragment for molecular confirmation of the isolates as *E. coli* simultaneously with the detection of the Class 1 integron. Class 1 integron was harboured by 57.1% of the isolates. All isolates contained *uidA* gene indicating that all species were *E. coli*. This study indicated that the primer pairs for Class 1 Integron and *E. coli* species-specific primers could successfully amplify both targeted amplicons of the desired sizes (Fig. 2).

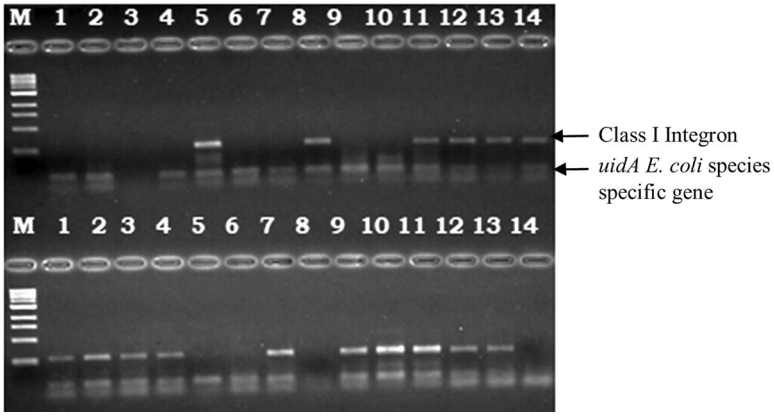

Fig. 2. Multiplex PCR specified for Class 1 Integron and *uidA*.

From left, lane M is 100 bp ladder from GeneOn (Taiwan). Lanes 1-14 in the top gel represent PCR products from isolates F1 to F14; in the bottom gel, Lanes 1-13 represent PCR amplicons from F15-F27 and Lane 14 is the negative control. The extensive use of aminoglycosides in animal feed has resulted in the prevalence of the gene cassettes *aadA1* or *dfrA17-aadA5* (El-Najjar et al. 2010). In *E. coli*, gentamicin resistance is most commonly mediated by AAC(3)-I, AAC(3)-II and AAC(3)-IV enzymes, and the increasing frequency of genes encoding these enzymes in the clinical isolates of human and animal origin has

been reported (Soleimani et al. 2014). Veterinary guidelines for the responsible use of antimicrobials have been developed by different organizations. Unfortunately in many countries those recommendations are not applied commonly in veterinary practice. The results also showed that companion animals in Bangladesh are an important reservoir of MDR *E. coli* strains. However, further investigations of antimicrobial resistance mechanisms in cattle and poultry *E. coli* populations in Bangladesh are required. Given the increasing existence of bacteria to drugs commonly used in small animal veterinary practice, it seems crucial that treatment of bacterial infections should be conducted according to antibiogram results. Only prudent, reasonable, and appropriate use of antimicrobials can minimize the emergence of MDR bacteria.

In a previous study PCR targeted at the conserved region of Class 1, 2 and 3 integrons was used (Zhang et al. 2019). The product of PCR was subsequently digested to identify the specific class of Integron present. The oligonucleotide primers Rfb and SLT-1 were used for targeting O157 and SLT-I genes of *E. coli*, respectively in previous studies (Imtiaz et al. 2013). In addition, multiplex PCR conditions were optimized for the detection of integrons in *E. coli* producing plasmid-mediated AmpC β-lactamases (Perez et al. 2002). The present study was able to validate the application of the primer pairs for *E. coli* species-specific identification and Class 1 integron detection.

The occurrence of antibiotic resistance and multidrug resistant *E. coli* in the animal gut is alarming as animal husbandry can lead to transmission of the resistance to human. The validation of the PCR primers previously used separately for species-specific identification of *E. coli* and the development of the multiplex PCR for the simultaneous detection of *E. coli* and Class 1 integron will be a useful tool in One Health management and implementation of the NAP. In the present study, Class 1 Integron was found in 61% of the examined isolates and the species-specific PCR worked successfully under the multiplex PCR conditions, thereby validating this PCR for future studies to be conducted in a relevant field. The outcome of the application of this PCR will help to promote the rational use of antibiotics in livestock in order to reduce or eliminate the risk from pathogenic antibiotic resistance bacteria originating from raw meat.

References

Ahmed SM, Naher N, Tune SNBK and **Islam BZ** (2022) The Implementation of National Action Plan (NAP) on Antimicrobial Resistance (AMR) in Bangladesh: Challenges and Lessons Learned from a Cross-Sectional Qualitative Study. Antibiotics (Basel) **11**(5): 690.

Briñas L, **Zarazaga** M, **Sáenz Y, Ruiz-Larrea F** and **Torres C** (2002) β-lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. Antimicrob. Agents Chemother. **46**(10): 156-63.

Cambray G, Guerout AM and **Mazel D** (2010) Integrons. Annu. Rev. Genet. **44**: 141-166.

CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 30th CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.

Cocchi S, Grasselli E, Gutacker M, Benagli C, Convert M and **Piffaretti JC** (2007) Distribution and characterization of integrons in *Escherichia coli* strains of animal and human origin. FEMS Immunol. Microbiol **50**(1): 126-32.

El-Najjar NG, Farah MJ, Hashwa FA and **Tokajian ST** (2010) Antibiotic resistance patterns and sequencing of class I integron from uropathogenic *Escherichia coli* in Lebanon. Lett. Appl. Microbiol. **51**(4): 456-461.

Godambe LP, **Bandekar J** and **Shashidhar R** (2017) Species-specific PCR based detection of *Escherichia coli* from Indian foods. 3 Biotech. **7**(2): 130.

Goldstein C, Lee MD, Sanchez S, Hudson C, Phillips B, Register B, Grady M, Liebert C, **SummersAO, White DG** and **Maurer JJ** (2001) Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. Antimicrob. Agents Chemother. **45**(3): 723-6.

Hall RM and **Collis CM** (1995) Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol. Microbiol. **15**(4): 593-600.

Holger C, Smith DEC, Blom H, Blau N, Bode H, Holzmann K, Pannicke U, Hopfner KP, Rump EM, Ayric Z, Kohne E, Debatin KM, Smulders Y and **Schwarz K** (2011) Dihydrofolate reductase deficiency due to a homozygous DHFR mutation causes megaloblastic anemia and cerebral folate deficiency leading to severe neurologic disease. Am. J. Hum. Genet. **88**(2): 226-231.

Imtiaz J, Hashmi I, Saeed A, Qazi I and **Arshad M** (2013) Development of PCR protocol for detection of *Escherichia coli* in drinking water. Water Resources Management VII; Brebbia, CA, Ed.; WIT PRESS: Southhampton, UK, pp. 225-232.

Leverstein-Van Hall MA, **Blok HEM**, **Rogier A**, **Donders T**, **Paauw A**, **Fluit AC** and **Verhoef J** (2003) Multidrug resistance among *Enterobacteriaceae* is strongly associated with the presence of integrons and is independent of species or isolate origin. J. Infect. Dis. **187**(2): 251-9.

Machado E, Cantón R, Baquero F, Galán JC, Rollán A, Peixe L and **Coque TM** (2005) Integron content of extended-spectrum-β-lactamase-producing *Escherichia coli* strains over 12 years in a single hospital in Madrid, Spain. Antimicrob. Agents Chemother. **49**(5): 1823-9.

Mazel D (2006) Integrons: agents of bacterial evolution. Nat. Rev. Microbiol. **4**: 608-20.

Pérez-Moreno MO, Centelles-Serrano MJ, Cortell-Ortolá M, **Ruiz J, Llovet-Lombarte MI, Jardí-Baiges AM** and **Fort-Gallifa I** (2009) Multidrug resistance related to class 1 integrons in human *Salmonella enterica* serotype Typhimurium isolates and emergence of atypical sul3-associated integrons. Int. J. Antimicrob. Agents **34** (4): 381-3.

Pérez-Pérez FJ and **Hanson ND** (2002) Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J. Clin. Microbiol. **40**(6): 2153-62.

Sabbagh P, Rajabnia M, Maali A, Ferdosi-Shahandashti E. Integron and its role in antimicrobial resistance: A literature review on some bacterial pathogens. Iran J Basic Med Sci. 2021 Feb; **24**(2): 136-142. doi: 10.22038/ijbms.2020.48905.11208. PMID: 33953851; PMCID: PMC8061329.

Schroeder CM, Zhao C, DebRoy C, Torcolini J, Zhao S, White DG, Wagner DD, McDermott PF, Walker RD and **Meng J** (2002) Antimicrobial resistance of *Escherichia coli* O157 isolated from humans, cattle, swine, and food. Appl. Environ. Microbiol. **68**(2): 576-81.

Soleimani N, Aganj M, Ali L, Shokoohizadeh L and **Sakinc T** (2014) Frequency distribution of genes encoding aminoglycoside modifying enzymes in uropathogenic *E. coli* isolated from Iranian hospital. BMC Res. Notes **7**: 842.

Van den Bogaard AE, London N, Driessen C and **Stobberingh EE** (2001) Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. J. Antimicrob. Chemother. **47**(6): 763-71.

www.who.int/news/item/17-10-2022-one-health-joint-plan-of-action-launched-to-address-health-threats-to-humans--animals--plants-and-environment**.**

White PA, McIver CJ and **Rawlinson WD** (2001) Integrons and gene cassettes in the *Enterobacteriaceae*. Antimicrob. Agents Chemother. **45**(9): 2658-61.

White PA, McIver CJ, **Deng YM** and **Rawlinson WD** (2000) Characterisation of two new gene cassettes, aadA5 and dfrA17. FEMS Microbiol. Lett. **182**(2): 265-9.

Zhang XY, **Ding LJ** and **Yue J** (2009) Occurrence and characteristics of class 1 and class 2 integrons in resistant *Escherichia coli* isolates from animals and farm workers in northeastern China. Microb. Drug. Resist. **15**(3): 323-8.

(Manuscript received on 30 March, 2023; revised received on 04 May, 2023)