Bangladesh J Microbiol, Volume 28, Number 2, December 2011, pp 84-87

Original Article

Detection of *ipaH* gene in Large Plasmid of *Escherichia coli* Isolated from Different Sources of Bangladesh

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(Received 01 October, 2011; Accepted 03 December 2011)

Large plasmid (more than 100 MDa) plays a significant role in *Escherichia coli* virulence as many virulent genes including the *ipaH* are encoded by large plasmids. Characterization of such large plasmid of *E. coli* from different sources based on *ipaH* gene has not yet been reported with success and credible reproducibility in Bangladesh. Forty *E. coli* isolates of environmental, clinical, avian, and mammalian sources were examined to detect the presence of large plasmids. Among them, plasmids recovered from 32 isolates were found to range between 100 and 145 MDa in size. Antibiotic susceptibility pattern of the isolates against 17 different antibiotics showed that most of them were resistant to more than 6 antibiotics and thus were designated multiple antibiotic resistant (MAR) strains. Out of 32 isolates, 10 were (3 clinical, 5 environmental, 1 avian and 1 mammalian source) found to possess a gene product of 423 bp size after PCR assay from plasmid DNA. Southern hybridization was carried out with *ipaH* probes and the results revealed the presence of band of correct size in both large plasmid DNA and in chromosomal DNA. Presence of the appropriate gene product in newly discharged environmental *E. coli* strains questions the credibility of using these bacteria as an indicator of water pollution in tropical countries like Bangladesh. The innovative approaches employed in this investigation regarding characterization of large plasmid based on the presence of *ipaH* gene in *E. coli* isolates from environmental, clinical, avian and mammalian sources opened up avenues for challenging research in future.

Keyword*: Large Plasmid, ipaH, Multi Drug Resistance (MDR), E. coli.*

Introduction

Bacterial plasmids are self-replicating, extrachromosomal replicons that are key agents of change in microbial populations[1]. Naturally occurring plasmids are able to promote the dissemination of a variety of traits including drug resistance, virulence, and the metabolism of rare substances[1]. Recombinant plasmids have been essential to the field of molecular biology, but the wild-type plasmids from which these tools were derived are often underappreciated[2]. *Escherichia coli* perhaps genetically the most versatile organism studied has been the source of many plasmid and phage mediated genes[3]. Most of the pathogenic genes such as *stx*1, *stx*2, *lt*, *st*, *hly*A, *agg*A, *saa*, *ast*A, *iuc*D, *cnf1*, *eaeA*, *bfp*A, *ial*, *ipaH*, *afa* etc are encoded by large plasmids[4,5].

E. coli has long been used as an indicator of fecal pollution. It has good characteristics of a fecal indicator, such as not normally being pathogenic to humans, and is present at concentrations much higher than the pathogens it predicts. However, many studies have suggested that *E. coli* may not be a reliable indicator in tropical and subtropical environments due to its ability to adapt and replicate in contaminated soils[6]. As large plasmids have been found to carry many virulent as well as drug resistant genes, an extensive characterization of large plasmids of environmental, clinical, and avian *E. coli* isolates at genotypic levels is indeed very important. Several virulent factors have been associated with *E. coli*, the most common being the ability to colonize and invade epithelial cells. This phenomenon is mediated by the 'invasion plasmid antigen H' (*ipaH*) gene which is carried on plasmid of 120 to 140 MDa[7,8]. The *ipaH* is one of the most important diarrheagenic genes that are involved in invasion and intracellular movement of colonic epithelium cells. Similar genes have also been detected in avian pathogenic *E. coli* as proved by colony blot hybridization[9]. Therefore, it indicates the necessity of detailed characterization of large plasmid of *E. coli* on the basis of its virulent *ipaH* genes.

In this study, isolation of large plasmid from environmental, clinical and avian *E. coli* isolates was performed and examined to investigate the presence of virulent *ipaH* gene. This would enable us to understand the role of large plasmid in *E. coli* from different sources in Bangladesh and also to conclude as to whether environmental *E. coli* would still be considered as a useful indicator organism.

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Materials and methods

Bacterial Strains

A total of 40 isolates of *E. coli* from different sources (environmental, clinical, avian, and mammalian) were taken from the stock of Department of Microbiology, University of Dhaka. *Shigella boydii* 12034 and *S. flexneri* 5a was used for plasmid molecular weight standard (140 MDa) and as positive control in PCR for *ipaH*.

Biochemical characterization

All isolates were previously identified as *E. coli* and these were reconfirmed by biochemical tests such as Simmon's Citrate test, Motility Indole Urease test, Kligler's Irom agar and Lysine decarboxylase test.

Antimicrobial susceptibility test

Bacterial susceptibility to antimicrobial agent was measured *in vitro* by utilizing the principle of agar diffusion as described by Bauer[10]. Commercially available discs (Oxoid, England) were used for the test. A total of 17 antibiotic discs were used in this study which are Polymixin B (300 unit), Cephalexin (30µg), Kanamycin (30µg), Nalidixic acid (30µg), Methicillin (5µg), Amoxycillin (25µg), Chloramphenicol (30µg), Tetracycline (30µg), Gentamycin (120µg), Azithromycin (15µg), Bacitracin (10 unit), Penicillin (10 unit), Sulphamethoxazole trimethoprim (25µg), Ciprofloxacin (5µg), Rifamycin (30µg), Ceftriaxone (30µg), Cefixime (5µg). Antimicrobial breakpoints and interpretation were taken from the CLSI standards (2006)[11].

Plasmid profiling

Plasmid DNA molecules are separated from chromosomal DNA for isolation and characterization of the plasmid DNA by the method described by Brinboim and Doly [12]. The method followed for agarose gel electrophoresis was done as Maniatis *et al.*[13]. The molecular weight of the unknown plasmid DNA was determined on the basis of its mobility through agarose gel and was compared with the mobility of the known molecular weight plasmid (supercoiled marker and 140 MD a plasmid from *Shigella boydii*12034 and *S. flexneri* 5a).

Extraction and purification of Chromosomal DNA

Chromosomal DNA of the test isolate and the reference strains was extracted and purified by the method described by Sambrook *et al*.[14]

Detection of ipaH by PCR assay

Primers used in this study were 5'-AGGTTAATCT-TTGCAGGGCT-3' and 5'-CAACAACCAGCTTACTGCCT-3'. Each PCR tube containing the appropriate mixtures were heated at 95°C for 3 minutes in the thermal cycler to ensure the complete denaturation of DNA templates. The PCR was then continued with the following programs, as denaturation for 1 minute at 94°C, , annealing for 1 minute at 55°C and extension at 72°C, for 1 minute. Thirty five (35) cycles of these segments were repeated with a final extension of 10 min at 72°C. PCR tubes were then stored at -20°C until further analysis.

Southern hybridization

Probe DNA for *ipaH* gene was prepared by PCR using chromosomal DNA of *S. boydii* ATCC 12034 as template and primers specific to *ipaH* gene. PCR product of probe DNA was purified by StrataPrep^{R} PCR purification kit. The protocol recommended by Roche (2004)[15] was followed for Digoxygenin (DIG)-dUTP labeling of probe DNA.

Results and Discussion

Biochemical characterization

The tests performed for all the 40 isolates of aquatic, clinical, avian and mammalian source were reconfirmed that the isolates possessed the biochemical characteristics typical of *Escherichia coli*. All the isolates were negative for citrate utilization and urease production but some variations were observed in motility and lysine utilization.

Antibiotic resistance patterns

Most of the 22 environmental *E. coli* isolates were 100% resistant to polymyxin B, cephalexin, penicillin G and gentamycin, 90-95% were resistant to methicillin, bacitracin, rifampicin, 63-77% were resistant to amoxycillin, tetracyclin, kanamycin, 22-45% were resistant to sulphamethoxasol trimethoprim, nalidixic acid and azithromycin, 9-18% were resistant to ciprofloxacin, cefixime, ceftriaxone, and chloramphenicol respectively. All the 4 avian and mammalian *E. coli* isolates were 100% resistant to polymyxin B, cephalexin, kanamycin, methicillin, bacitracin, penicillin G, only one (25%) was resistant to sulphamethoxasol trimethoprim, nalidixic acid, 50-75% were resistant to azithromycin, rifampicin, cefixime. All the isolates were found to be completely sensitive to six of the antibiotics (amoxycillin, ciprofloxacin, ceftriaxone, tetracyclin, gentamycin and chloramphenicol). All the 6 clinical isolates were resistant to polymyxin B, cephalexin, kanamycin, methicillin, amoxycillin, bacitracin, penicillin G and rifampicin. However, 34-50% of the isolates were resistant to tetracycline, sulphamethoxasol trimethoprim, ceftriaxone, 67-84% of the isolates were found to be resistant to ciprofloxacin, nalidixic acid, azithromycin and cefixime respectively but most of the clinical strains were sensitive to gentamicin.

Plasmid profile analysis and detection of ipaH gene in large plasmid by PCR

Plasmid profile of all the 40 isolates were compared with supercoiled 1 kb ladder and with two 140 MDa plasmid positive *Shigella* isolates (*Shigella boydii* 12034 and S. *flexneri* 5a). Large plasmid was recovered from 32 isolates, only 3 isolates (1 environmental and 2 avian isolates) did not contain any detectable plasmid (Fig. 1). PCR analysis revealed that out of 32 isolates containing large plasmid 10 isolates (5 environmental, one avian, one mammalian and 3 clinical) gave the appropriate product of the *ipaH* gene (Fig 2).

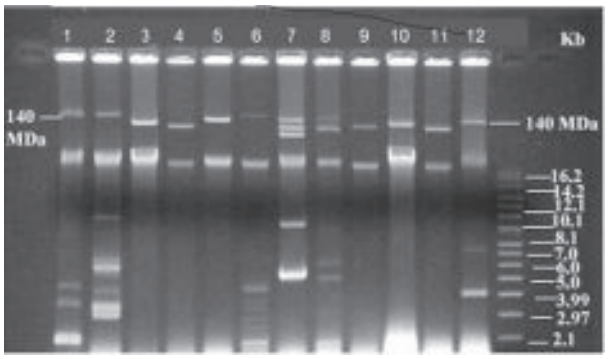

Figure 1. *Plasmid profile of isolates analysed by (0.8%) agarose gel electrophoresis after staining with ethidium bromide and the DNA band were visualized by UV transilluminator. (E=environmental, C= clinical, M= mammalian A= avian isolates) Lane 1: Shigella boydii 12034, Lane 2: C3, Lane 3: E15, Lane 4: E14, Lane 5: A2, Lane 6: C2, Lane 7: E10, Lane 8: C1, Lane 9: E23, Lane 10: M1, Lane 11: E8, Lane 12: Shigella flexneri 5a, Lane 13: Super coiled Marker*

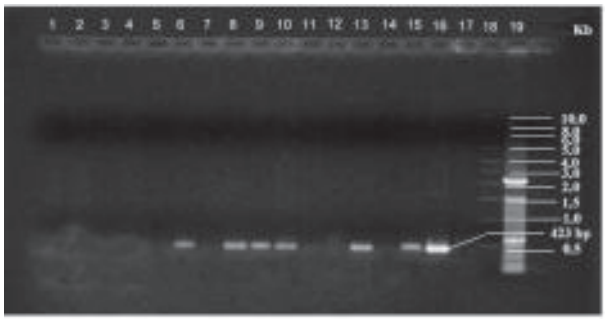

Figure 2. *Agarose gel electrophoresis showing PCR amplification products of the ipaH gene (E=environmental, C clinical, M= mammalian A= avian isolates) Lane 1: E25, Lane 2: A4, Lane 3: E6 Lane 4: E3, Lane 5: E21, Lane 6: E14, Lane 7: C6, Lane 8: E15, Lane 9: E12, Lane 10: A2, Lane 11: C7 Lane 12: E16, Lane 13: C3, Lane 14: E4, Lane 15: C2, Lane 16: Shigella boydii 12034 (positive control) Lane 17: Negative control, Lane 18: 1 Kb ladder, Lane 19: 100 bp ladder*

Southern hybridization

Southern hybridization was carried out to confirm the presence of *ipaH* gene that was revealed in the *ipaH*-specific PCR reactions. The *ipaH* probes were used in the hybridization reaction with DIG and *ipaH* PCR positive isolates showed bands in the membrane after hybridization with *ipaH* probe. Hybridization patterns for *ipaH* genes with two positive controls can be seen in the Figure 3.

E. coli have large plasmids that encode many diarrhea-associated virulent genes including *eae, stx, aggR, est, slt, ipaH*[16]. The invasion plasmid antigen H (*ipaH*) genes are carried on large plasmid of 120 to 140 MDa in *E. coli*. The present study was

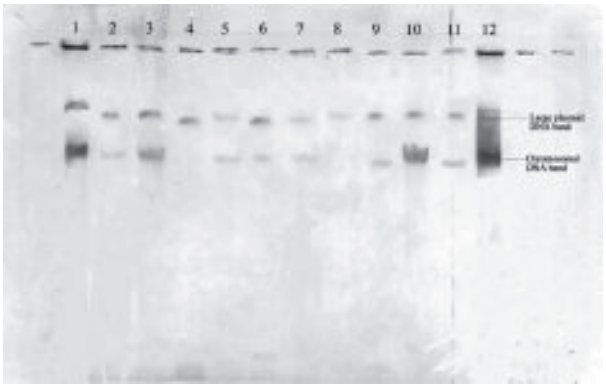

Figure 3. *Determination of the presence of ipaH gene by Southern blot hybridization. (E=environmental, C clinical, M= mammalian A= avian isolates) Lane 1: Positive control (Shigella boydii 12034), Lane 2: C3, Lane 3: E15, Lane 4: E14, Lane 5: A2, Lane 6: C2, Lane 7: E10, Lane 8: C1, Lane 9: E23, Lane 10: M1, Lane 11: E8, Lane 12: Positive control (Shigella flexneri 5a).*

focused on the isolation of large plasmid from *E. coli* of environmental, clinical, avian and mammalian origins followed by detection and comparison of the presence of *ipaH* gene in the large plasmid.

One of the major interests of this research was to find out any possible correlation between the antibiotic sensitivity pattern and plasmid profile analysis among the environmental, clinical, avian and mammalian isolates. It was observed that, all the 6 clinical isolates were completely resistant to at least 10 antibiotics or more out of 17 different antibiotics whereas 22 environmental isolates were found to be resistant to the same antibiotics as well. However, clinical isolates were found to be sensitive to very few antibiotics in comparison to the environmental isolates. Environmental isolates revealed that these have become multiple antibiotics resistance possibly mediated by the presence of large conjugative plasmid in environmental isolates. Multiple antibiotic resistance among the environmental strains studied, could not reveal any specific host preference/association. But it suggests the presence of diverse type of *E. coli* strains in the environment. It further lends support that *E. coli* has probably become adapted and/or survived in the environment in adverse conditions.

Many virulent and antibiotic resistance genes are encoded by large plasmids. Therefore, large plasmids (more than 100 MDa size) may play a significant role in *E. coli* virulence. In this study, 22 environmental, 3 avian and 1 mammalian isolates possessing large plasmid (ranged between 118 and 140 MDa) were found to be resistant to at least 8 antibiotics or more, while the 6 clinical isolates containing plasmids (ranged between 120 and 145 MDa) were resistant to 10 antibiotics or more. So, it suggests that large plasmid might contribute to resistance against such large number of antibiotics.

In the present study, it was revealed that out of 32 isolates possessing large plasmid, 10 isolates (5 environmental, 3 clinical,

1 avian and 1 mammalian isolates) contained the *ipaH* gene product (Fig. 2). These isolates possessing the *ipaH* gene product was observed within the large plasmids that ranged between 120 and 140 MDa. Since sequencing could not be performed for the specific gene product, Southern hybridization was done to confirm the presence of the *ipaH* gene within the isolates (Fig 4). Earlier it has been described in a report that *ipaH* is present in multiple copies on both the plasmid and the chromosome of *Shigella* spp.[17,18]. In this study, 8 isolates produced 2 hybridizing fragments both for large plasmid and chromosomal DNA. But 2 isolates produced only 1 hybridizing fragment of large plasmid DNA. Thus, Southern hybridization of the plasmid DNA from *ipaH* positive isolates justifies the results of PCR for the detection of *ipaH* gene.

Conclusion

Finally, it can be concluded from this investigation that *ipaH* gene was found to be present in large plasmid of *E. coli* but that all the large plasmids do not encode *ipaH* gene. The presence of *ipaH* gene in chromosome of *E. coli* has not yet been reported in Bangladesh. So, the finding of the present work demonstrated that *ipaH* gene product was present in both the large plasmid and in the chromosome of *E. coli*. This study also suggests that large plasmid might contribute to resistance against large number of antibiotics to those isolates. Additional experiments for virulence assay involving Congo red binding ability and Serenity test should be done in future in order to confirm the large plasmid mediated invasiveness property for these isolates. Sequencing of the PCR products will further confirm the presence of this gene in large plasmids of *E. coli*. This could change the notion of using *E. coli* as a recent fecal indicator of water quality.

Acknowledgements

The research was a joint collaboration between the Department of Microbiology, University of Dhaka, Bangladesh, and the Department of Biology, Bergen University, Norway. The authors are grateful to NUFU, Norway for the funding of this project.

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