Genotypic Detection and Characterization of Adhesins in Clinical Escherichia coli Isolates

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Authors’ contributions

This work was carried out in collaboration among all authors. Author MYI designed the study and carried out the selection of isolate, DNA extraction, PCR amplification of virulence genes, agarose gel electrophoresis, gel image visualization and documentation and sequencing analysis at the university of Abertay Dundee, UK. Authors HY and IM collected and processed the samples by culture, macroscopy/plate reading, Gram staining, microscopy, biochemical identification tests and antimicrobial susceptibility testing. Author HSM, performed the statistical analysis, wrote the protocol, and the first draft of the manuscript. Authors AFU, EBA and AU respectively managed the analyses and the literature searches. Author YYD managed and supervised the molecular protocols at the Abertay University, Dundee, UK (formerly). All authors read and approved the final draft of the manuscript.

ABSTRACT

Background of Study: Many virulence determinants contribute to the pathogenicity of Gram negative bacteria, like Escherichia coli, which is the most common cause of many infections worldwide such as urinary tract infection (UTI), profuse diarrhoea and septicemia.
Aim: To determine the genotypic characteristics of adhesin-producing E. coli isolates from clinical specimens.
Place and Duration of Study: Conducted at the Infectious diseases hospital Bayara, Bauchi state, Nigeria, between February to March, 2019.

Methods: A total of twelve (12) Gram negative bacterial isolates were selected based on the ability to grow on Luria-Bertani (LB) agar medium containing 100 µg/ml ampicillin. The isolates were from urine, stool, and blood specimens. The isolates were screened for multidrug resistant pattern according to Kirby-Bauer disc diffusion method. Adhesion factors, Fimbrial adhesin (fimH) and Invasive plasmid adhesin (ipaH) was genotyped by conventional PCR and sequenced.

Results: All the isolates were resistant to Ampicillin, Cephapoloth, Erythromycin, Fusidic acid, Novobiocin and OXacillin, but sensitive to Augmentin, Colisine sulphate and Imipenem. Presence of fimH and ipaH genes were observed in nine isolates that expressed strong relationship with. Multidrug resistance (MDR). The fimH was the most prevalent found in urine, stool and blood isolates. Most of the adhesion genes sequence (61.8%) in this study had significant alignment (95 to 100% homology) with E. coli genome in the NCBI database.

Conclusion: This study revealed the role of adhesin as virulence markers in MDR Gram negative bacteria and FimH is one of the commonest gene in MDR E. coli pathotypes.

Keywords: Multidrug resistance; adhesion factors; fimbriae adhesion; invasive plasmid adhesion; genome.

1. INTRODUCTION

Acquisition and dissemination of virulent traits represent a survival advantage to bacterial pathogens. Escherichia coli is a common non-invasive commensal bacteria established as etiological agent of various human infections. It is the most prominent cause of infectious diseases that span from the gastrointestinal tract to extra-intestinal sites such as genital and urinary tract infection, septicaemia, and neonatal meningitis [1]. The E. coli that cause these diseases have specific pathogenic attributes that enable them to cause disease [2].

Virulence factors are molecules and structures expressed by microbes like bacteria that enable them to achieve pathogenicity [2]. They are very often responsible for causing disease in the host and converting non-pathogenic bacteria into dangerous pathogens [2]. In bacteria, virulence factors are often encoded on plasmids and chromosomes and can easily be spread through horizontal gene transfer [3].The virulence of the individual strains in a given infection is determined by the presence and the actual expression of the virulence genes which are present in them and also by the environmental conditions in the host [4].

Escherichia coli can cause infections due to its ability to the acquisition of mobile genetic factors carrying many virulence genes. Adhesins, iron uptake, toxins and capsules are the most common virulence factors responsible for attachment, adherence and invasion in the host and then lead to infection [5]. Pathogenicity of E. coli is due to the presence of many virulence genes, located on chromosomes or plasmids or both that encodes important virulence factors, if present on the chromosome, these genes are typically found in specific regions called pathogenicity islands.

Adherence to solid substrates is a property common to many pathogenic microorganisms, including viruses, gram-positive and gram-negative bacteria. By attaching to host structures, microbial pathogens avoid being swept along by the normal flow of body fluids (blood, urine, intestinal contents) and eliminated, although host cells with adherent bacteria can be shed, thereby eliminating the organisms despite attachment. Adherence of E. coli isolates to uroepithelial cells is used to differentiate between uropathogenic and faecal strains. It was suggested that uropathogenic strains may present a mean of 20 bacteria/cells or more, while faecal strains may present a mean of about 7 bacteria/cell. UPEC strains causing UTI typically agglutinate human erythrocytes despite the presence of mannos (mannose-resistant haemagglutination, MRHA) and adhere to human uroepithelial cells. Also, adherence to uroepithelial cells is usually unaffected by mannos (mannose-resistant adherence) and is more common among strains exhibiting MRHA than among those exhibiting only mannos-sensitive haemagglutination [6].

Type I pili play a role in gastrointestinal disease, although many enteric E. coli pathogens and non-pathogenic faecal isolates produce this type of pili. Adherence mediated by type I fimbriae is blocked by solutions of D-mannose or α-
methylmannoside and by concanavalin A (a lectin that binds to mannoses residues) but not by solutions of other monosaccharide or their derivatives [7]. The type I-Fimbrial receptor is probably an extended structure, since mannoses with α-linked aromatic group, trisaccharides, and branched oligosaccharides that contain (α-1-3) – linked mannoses are better inhibitors of type I-Fimbrial binding than D-mannose or α-methylmannoside alone. Receptors for type I fimbriae are present on erythrocytes from many species. Type I-fimbriated bacteria adhere to human buccal epithelial cells, intestinal cells, and vaginal cells, suggesting a possible role for type I fimbriae in E. coli colonization of the mouth, gut, and vagina. Receptors for type I fimbriae are present in blood vessel walls and in the muscular layers but not the epithelium of the human bladder [6, 8].

Type I pili have composite structure of 7 nm in width and 1 - 2 microns in length and consist of long rigid rod and a distal thin fibrillar structure. E. coli fimbriae, type I fimbriae are encoded by a gene cluster fim that includes genes for a structural subunit, an adhesin, several accessory proteins (involved in subunit transport and assembly and in anchoring assembled fimbriae), and regulatory proteins. FimH is the adhesin protein responsible for binding to mannosylated glycoproteins and is located at the distal tip of the heteropolymeric type I pilus rod, which is predominantly constituted by FimA subunits. FimG and FimH are minor components that are probably needed as adaptors, initiators or terminators. FimC is the chaperone and FimD outer membrane usher. FimH mediates E. coli binding to mannose-containing glycoprotein receptors - uroplakins, which are located on the luminal surface of the bladder epithelial cells [7, 8].

Previous studies describing the clinical significance of virulent E. coli infections have focused mainly on phenotypic characterization of isolates from cases of UTIs [9]. Measuring a phenotype in vitro does not always correlate with in vivo expression and may underestimate the presence of adhesion factor in a particular strain [10]. Phenotypic methods cannot differentiate between the specific genes responsible for pathogenicity. Molecular studies of pathogenic bacteria hold significant promise in understanding the virulence factors thereby identifying coordinates highly relevant in the development of treatment strategies. This study is therefore aimed to determine the genotypic characteristics of adhesin producing E. coli isolates from clinical specimens.

2. MATERIALS AND METHODS

2.1 Selection of Bacterial Isolates

Twelve (12) clinical bacterial isolates phenotypically confirmed positive for virulence factors; type 1 fimbriae and Fimbrial adhesin were selected out of 153 multidrug resistant and beta-lactamase producers, from our previous study [11]. The isolates were also screened for the molecular analysis by growing on Luria-Bertani (LB) agar medium containing 100µg/ml ampicillin (Melford Laboratories, UK), as described by [12].

2.2 Screening of Isolates for Multidrug Resistance Characteristics

The isolates were screened for antimicrobial susceptibility pattern, multidrug resistant characteristics on Iso-sensitest agar (ISA) media (Oxoid, UK) according to Kirby-Bauer disc diffusion methods as described by [12]. The following commercial discs were used: Combined discs M13/M14 rings (Mast Diagnostics, UK): Chloramphenicol (5 µg), Erythromycin (5µg), Fusidic acid (10 µg), Oxacillin (5ug), Novobiocin (5 µg), Penicillin (1I.U), Streptomycin (10µg), Tetracycline (25 µg), and M14 rings: Ampicillin (10 µg), Cephalothin (5ug), Colistin sulphate (25 µg), Gentamycin (10 µg), Sulphatrad (200µg), and Cotrimoxazole (25 µg). Single discs (Oxoid): Amoxycillin-Clavulanate/Augmentin (30 µg), Cefuroxime (30 µg), Ceftriaxone (30 µg), Cefotaxime (30 µg), Ciprofloxacin (10µg) and Imipenem (10 µg). The diameter zone of inhibition were measured in millimetre and results were interpreted according to British society for antimicrobial chemotherapy (BSAC) guidelines [13]. Escherichia coli ATCC 35401 was used as positive control, while E. coli K-12 DH5α as negative control.

2.3 Genotypic Detection of Adhesin

2.3.1 Extraction of DNA from bacterial isolates

The clinical isolates were grown for 24 hours on Luria-Bertani (LB) agar plates (containing 100 µg/ml ampicillin). Chromosomal DNA was isolated from overnight bacterial culture by boiling method as described by [12]. A loopful of cells from a single colony was transferred to
100 µl of sterile/double distilled water and the mixture was boiled for 10 min, at 95°C in water bath to lyse the cells. The cell lysate were then centrifuged briefly (10s at 10,000 rpm). The supernatant was carefully pipetted into fresh tube and kept at -20°C for further use. A 5µl of the sample was used for the PCR reaction [14]. Plasmid DNA was isolated from the samples using Qiagen miniprep protocol by alkaline lysis method according to manufacturers’ instructions.

2.3.2 PCR amplification of adhesion

Adhesion factors were amplified using *ipaH* and *fimH*, specific primers (Integrated DNA Technologies, USA). PCR was performed in a final reaction volume of 25 µl, which comprises of 5.5 µl sterile distilled water, 1 µl (each) reverse and forward primers, 12.5 µl of Master mix, Taq DNA polymerase, and 5 µl of bacterial lysate (supernatant with template DNA). Amplifications was performed with the G-Storm thermocycler GS0001 (ThermoFisher Scientific, UK). PCR amplification start with initial denaturation at 94°C for 5 minutes, then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 1 minute. A final extension at 72°C for 10 minutes was conducted, as described by [15]. A molecular marker 1 kb DNA Hyperladder (Bioline, UK) was used to assess the PCR product size. The product was separated by gel electrophoresis on 1.0% agarose, stained with gel red and the image was captured digitally with UV transillumination (Syngene). The product size were estimated using 1kb DNA Ladder (Bioline, UK). The PCR products was purified and stored at 4°C, before sequencing. The characteristics of the primers used and the expected amplicon size are given as follows:

| Gene  | Primer | Oligonucleotide sequence (5’ - 3’) | Size (bp) | Reference |
|-------|--------|-----------------------------------|-----------|-----------|
| IpaH  | IpaH-F | TGAAAGACGTAGGCCTCT               | 423       | [16]      |
|       | Stx1-R | CCGTGAAATTCATTCT                 |           |           |
| FimH  | FimH-F | GAAGAGGTGGTATTAACTTATTG          | 508       | [17]      |
|       | FimH-R | AGAGCCGCTGTAGAAACTTGG            |           |           |

3. RESULTS AND DISCUSSION

3.1 Multidrug Resistant Characteristics of the Bacterial Isolates

Multidrug resistance in bacteria is most commonly associated with the presence of plasmids which contain one or more resistance genes, each encoding a single antibiotic resistance phenotype. It has been shown that antibiotics therapy can select for antibiotic resistant strains in the enterobacterial flora and plasmid-mediated antibiotic resistance can spread in a population subjected to heavy antibiotic therapy [18].

In the present study, (Table 2), all the twelve isolates (100%) are resistant to Ampicillin, Cephalothin, Erythromycin, Fusidic acid, Novobiocin and Oxacillin. Eleven isolates (91.7%) are resistant to Chloramphenicol, Cotrimoxazole, Streptomycin, Sulphatrim and Tetracycline. Eight isolates (66.7%) are resistant to Ciprofloxacin. Seven isolates (58.3%) are resistant to Cefotaxime, Ceftriaxone Cefuroxime and Gentamycin. Nine (9) isolates (75%) are sensitive to Colistin sulphate (100%), Imipenem (100%), and Augmentin (75%). The *E. coli* isolates U03, U04, S05, S06, S07, B09, U10, U11 and S12 were sensitive to Augmentin and at least five of the isolates are also sensitive to Cefotaxime, Ceftriaxone, Cefuroxime and Gentamycin. The result showed higher resistance of the isolates to beta-lactams like ampicillin which alarms us that such drugs should no longer be used as first line of treatments in this area, as it used to be in the previous decades. These results of ampicillin-resistant *E. coli* are congruent to the report of [19], who found 100% resistance of their *E. coli* isolates to ampicillin.

The greater prevalence of resistance to common antibiotics was reported by Mohajeri et al. (2014) [10], where maximum number of isolates (100%) was resistant to ampicillin, carbenicillin and ceftazidime and the lowest to chloramphenicol.
Table 2. Antimicrobial susceptibility profile of isolates from this study

| Antimicrobial agent (µg)       | Number of isolates (n=12) and susceptibility pattern (%) |
|-------------------------------|---------------------------------------------------------|
|                              | No. (%) | No. (%) |
|                              | Sensitive | Resistant |
| Ampicillin (10)              | 00(0.0) | 12(100) |
| Augmentin (30)               | 03(25.0) | 10(75.0) |
| Ceftriaxone (30)             | 05(41.7) | 07(58.3) |
| Cefuroxime (30)              | 05(41.7) | 07(58.3) |
| Cefotaxime (30)              | 05(41.7) | 07(58.3) |
| Cephalothin (5)              | 00(0.0) | 12(100) |
| Chloramph. (10)              | 01(8.33) | 11(91.7) |
| Ciprofloxacin (5)            | 04(33.3) | 08(66.7) |
| Cotrimoxazole(25)            | 01(8.33) | 11(91.7) |
| Colistin sulph. (25)         | 12(100) | 00(0.0) |
| Erythromycin (25)            | 00(0.0) | 12(100) |
| Fusidic acid (5)             | 00(0.0) | 12(100) |
| Gentamycin (10)              | 05(41.7) | 07(58.3) |
| Imipenem (10)                | 12(100) | 00(0.0) |
| Novobiocin (5)               | 00(0.0) | 12(100) |
| Oxacillin (5)                | 00(0.0) | 12(100) |
| Penicillin G (1 I.U)         | 00(0.0) | 12(100) |
| Streptomycin (10)            | 01(8.33) | 11(91.7) |
| Sulphatriad (200)            | 01(8.33) | 11(91.7) |
| Tetracycline (30)            | 01(8.33) | 11(91.7) |

N.B: Zones of inhibition was interpreted according to BSAC, 2013 guidelines.

Fig. 1. Fimbria adhesin gene PCR products (chromosomal DNA) agarose gel image. Lane 01 to 12, are isolates that harbours fim1 gene with expected amplicon size of 508 bp [17] (Jalali et al., 2015). Lane M is molecular marker (1kb DNA ladder, Bioline, UK). Lane C is a control strain, ATCC 3450 (Virulent E. coli). The figure shows that the isolates, U01, U03, S05, S06, S07, U08, B09, U10, U11 and S12, harbour FimH gene.

Fig. 2. Invasive plasmid adhesion (ipaH) gene PCR products agarose gel image. Lane 01, 02, 07, 09 and 11, are isolates that harbours ipaH gene with expected amplicon size of 423 bp [17]. Lane M is molecular marker (1kb DNA ladder, Bioline, UK). The results shows that the isolates, U01, U02, S07, U08, B09, and U11 harbours ipaH gene.
3.2 Genotypic Characteristics of Adhesins

The ability of some bacteria like E. coli to cause diseases is determined by harbouring and acquisition of some pathogenic attributes [22, 23]. For example, presence of cytotoxins, Fimbrial adhesions and invasive plasmid adhesion in Gram negative bacteria is responsible for febrile UTI in some women and diarrhoeal cases of medical importance [2]. The results of this study show that adhesion genes are more commonly found among gastrointestinal bacteria (stool isolates) than in other cases such as UTI.

Tarchouna et al. [24] reported frequent occurrence of fimH and haemolysin in patient with UTI. Adherence through fimbriae is an important property of uropathogenic E. coli. The degree of severity depends on the virulence of the responsible strains and on the susceptibility of the host [25]. A better cognition of the virulence characteristics of the microorganism causing infection will permit the clinician to anticipate the evolution of infection in the host.

This study found that the urine isolates with this high prevalence of adhesin can be a major causative agent for UTIs in humans in our area. In spite of the previous studies, this results showed that the urine isolates have a different virulence profile. These differences in prevalence of adhesion genes showed that the properties is closely depending on geographic region and even weather conditions of each regions. It seems that the epidemiology and prevalence of adhesion factors of urine isolates from patients with UTI is different in this population. It was also confirmed that FimH is important in pathogenesis and the pathogenicity of these gene encoding isolates. It also depends greatly on the ability of the gene to switch between conformations and this is dependent on the different alleles that can be expressed by this gene. It is well documented that type 1 pil are important for the invasion and persistence of the UPEC in the urinary bladder after its colonization. The colonization is enhanced by Fimbrial adhesin FimH, encoded by fimH gene which recognizes certain α1β3 integrins [25, 26]. Aljanaby and Alfaham [5] reported that the fimH adhesion gene was present in 45(90%) of their isolates, 23 isolates from urine, 15 isolates from heavy diarrhoea and 7 isolates from blood. Yun et al. [27] reported that feoB and fimH were the most prevalent virulence genes in E. coli strains isolated from human with UTI.

Adherence to host cells is due to a plasmid such as ipaH that encodes genes required for invasion, cell survival and apoptosis of macrophages [10]. The adhesion genes in E. coli are often located on transmissible genetic elements that can be transferred to E. coli recipient strains. These virulence determinants give each pathotype the capacity to cause a clinical syndrome with distinctive epidemiologic and pathologic characteristics [28] and are therefore ideal targets for the determination of the pathogenic potential of any given E. coli variant. Shiga toxin (stx) is one of the major virulence factors involved in E. coli O157:H7 pathogenesis based on immune-reactivity, toxins are classified as either Stx1 or Stx2, which damage intestinal epithelial cells and kidneys, causing haemorrhagic colitis and haemolytic uremic syndrome, respectively [29]. The stx gene in E. coli O157:H7 is associated with a prophage, and different subtypes of shiga-toxin are identified [25].

The prevalence of ipaH and stx genes in our studies was low. However, these virulence genes are usually associated with occurrence of diseases. A study in Egypt by [30], showed similar distribution of virulence genes. This indicates that some social and environmental factors may contribute in the virulence pattern of E. coli in different communities. The negative isolates for some of the genes in our study may be part of the normal flora that lack these virulent genes or may be due to the possibility of corresponding gene mutations, as negative test
results does not always indicate the absence of the corresponding operon while a positive result usually confirms the presence of the virulence genes. When antibiotic resistance was tested among the virulent isolates, a significant relation was observed, where most of the isolates harbouring the virulence genes were multidrug resistant [17].

In this study, the fimH gene was the most common virulence gene found in urine, stool and blood isolates. Prevalence of these genes vary according to the phylogenetic groups, clinical conditions of host and geographical localization. The results showed a higher frequency of fimH compared with the rest ipaH genes, which may indicated an essential role of the adherence genes in Escherichia coli causing UTI. These results agree with previous reports by [17] and [25] in their independent studies which emphasize the predominance of fimbriae among the UPEC strains. The patients from whom isolates had fimH, probably suffered from cystitis and descending infection [17]. This was the observation in this study, where most of the patients had various cases of UTI. Previous studies have established that fimH is most frequent in isolates from a variety of forms of UTI [31, 32]. After analysis of positive urine samples for the presence of E. coli in patients with UTIs, it was recognized that fimH gene had the highest (79.7%) prevalence [33].

3.3 Adhesin Gene Sequences with Significant Alignment

Escherichia coli is a common and widespread bacterium associated with various infectious diseases of both intestinal and extraintestinal sites. This is due to acquisition and dissemination virulence genes that determined their pathogenicity. The worldwide burden of these diseases is staggering, with hundreds of millions of people affected annually. Eight E. coli pathovars have been well characterized, and each uses a large arsenal of virulence factors to subvert host cellular functions to potentiate its virulence [34].

FimH genes from isolates S12 shows a high significant sequence alignment at 97% homology with the strain (Table 3), E. coli CFSAN029787 DNA, complete genome (CP011416.1).The strain CFSAN029787 is a serotype O96:H19, an enteroinvasive E. coli that belongs to phylogenetic group B1and was first isolated in Italy [35]. Enteroinvasive Escherichia coli is a unique group of disease-causing bacteria that have a virulence mechanism most similar to that of Shigella bacteria, involving the invasion of intestinal epithelial cells. In contrast, the other pathovars of E. coli do not invade host cells and, instead, typically associate with the surface of the host cell and secrete or translocate virulence factors onto or into the cell [35]. The virulence of Shigella and EIEC bacteria has been attributed to genes associated with mobile genetic elements including pathogenicity islands and a virulence plasmid, pINV. The pINV plasmid is required for invasion of intestinal epithelial cells and encodes a type III secretion system (T3SS) and many associated effectors [36].

Enteropathogenic Escherichia coli (EPEC) were the first adhesin-producing E. coli strains recognized as important pathogens in diarrheal diseases [37]. Even today, EPEC strains are the major bacterial cause of neonatal and infantile gastroenteritis throughout the world, especially in developing countries [37]. These bacteria has been recognized to exhibit a great pathogen-related risk of death in infants, usually younger than a year [38]. Previous studies have shown that morbidity and mortality rates due to these E. coli strains are quite significant, especially in developing countries [39].

Virulence is due to a plasmid such as ipaH that encodes genes required for invasion, cell survival and apoptosis of macrophages [10]. The adhesin genes in E. coli are often located on transmissible genetic elements that can be transferred to E. coli recipient strains. These virulence determinants give each pathotype the capacity to cause a clinical syndrome with distinctive epidemiologic and pathologic characteristics [28] and are therefore ideal targets for the determination of the pathogenetic potential of any given E. coli variant. The ipaH gene sequence in this study shows low significant alignment, less than 95% homology.

The prevalence of ipaH genes in our study was low. However, these virulence genes are usually associated with occurrence of diseases. A study in Egypt by [30], showed similar distribution of this gene. This indicates that some social and environmental factors may contribute in the virulence pattern of E. coli in different communities. The negative isolates for some of the genes in our study may be part of the normal flora that lack these virulent genes or may be due to the possibility of corresponding gene
4. CONCLUSION

This study found that *FimH* is the most prevalent gene among MDR of *E. coli* pathotypes. It revealed the ability of *E. coli* to adapt and survive in different tissues by producing adhesins and developing drug resistance. The expression of adhesion factors may depend on the need and it varies in different kinds of infections. Drug resistance is on the increase among human pathogenic *E. coli*. Proper selection of antibiotics for treatment should depend on the susceptibility test outcomes. Most of the isolates in this study are resistant to commonly used antibiotics. Therefore, prescription of such Carbapenems as Imipenem is advocated in the events of these therapeutic failures.

**COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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