VIRULENCE DETERMINANTS OF ESCHERICHIA COLI STRAINS ISOLATED FROM SURGICAL SITE INFECTIONS AT SELECTED HOSPITALS IN SYRIA

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Escherichia coli is one of the most commonly isolated pathogens from surgical site infections (SSIs) that accounts for significant morbidity and mortality, especially with high virulent strains. The purpose of this study was to evaluate the virulence determinants of E. coli strains isolated from surgical sections. 51 E. coli isolates were assessed for biofilm formation, mannose-sensitive or -resistant hemagglutination, capsule, hemolysin and siderophores production. Antibiotic susceptibility against 14 common antibiotics was performed. As a result, 25 strains displayed 5 virulence factors and multiple drug resistance towards more than 11 antibiotics. DNA and plasmids were extracted from the 25 virulent isolates. PCR was used to investigate virulence genes on DNA encoding adhesins (fimH¹, mrkD), hemolysin (hlyA), siderophores (entB: enterobactin, iutA: aerobactin, trp-1: yersiniabactin- which was encoded on (HPI) high-pathogenicity island). MrkD, iutA and hlyA genes were also screened on extracted plasmids. The most common virulence genes were iutA (25/100%), mrkD (24/96%), entB (23/92%) and fimH-1 (21/84%). Irp1 was found at moderate rates (15 /60%) and at lower prevalence, was gene hlyA (2 / 8%). Plasmids were found in 16/25 strains. MrkD and iutA were present in 10 /16 plasmids, whereas none of them harbored hlyA. In conclusion, most E. coli isolates harbored high frequencies of (fimH1, mrkD, entB and iutA) which seem to be at the basis of pathogenicity. However, some strains, which carry HPI and have virulence plasmids can account for even more real threat if they spread among other Enterobacteriaceae members in surgical sections.

INTRODUCTION

Surgical site infections (SSIs) are one of the most common types of nosocomial infections and major global health problem¹². SSIs can lead to increasing the patients' health care cost and hospital stay, morbidity, and mortality²³. These infections are usually caused by microorganisms, especially gram positive and gram negative bacteria that may enter the patient's wound during or after the surgery³. One of the most commonly isolated gram negative bacteria according to bacteriological studies is Escherichia coli⁴.

E. coli is a facultative anaerobic bacterium belonging to the Enterobacteriaceae³. Some E. coli strains may cause intestinal and extra-intestinal infections in humans. The strains that cause gastroenteritis are commonly referred to as the diarrheagenic and are subdivided into six pathotypes, i.e. enterotoxigenic E. coli (ETEC), enteropathogenic E. coli (EPEC), enterohemorrhagic E. coli (EHEC), enteroaggregative E. coli (EAEC), enteroinvasive E. coli (EIEC), and diffusely adhering E. coli (DAEC⁶⁷). Whereas extraintestinal pathogenic E. coli (ExPEC) group includes uropathogenic E. coli (UPEC), neonatal meningitis E. coli (NMEC),
sepsis-associated E. coli (SEPEC), and avian pathogenic E. coli (APEC)⁶.

E. coli strains’ Pathogenicity is related to the virulence factors that it has, such as: adhesins, toxins, siderophores, lipopolysaccharides, capsules, and invasins⁸. A combination of factors will make the bacterium more virulent and cause more infections¹⁰.

The acquisition of virulence genes which encode to virulence factors is believed to increase the pathogenicity of bacteria and the severity of infection with the great possibility of therapy failure¹¹. These virulence factors are encoded on pathogenicity islands (PAIs) and plasmids⁸. These plasmids can be either integrated into the chromosome or replicate independently as extrachromosomal elements and later on they can be transferred to other species contributing to inter- intra-species variability in genomic contents.¹⁰

Surface virulence factors (adhesins) such as type 1, type 3 fimbriae (which are encoded by fimH and mrkD genes respectively) are very important as the main attachment factor⁷. α-haemolysin (hlyA) is an important lipoprotein toxin that constitutes a part from adhesins⁹. Other important virulence factors are siderophores, low-molecular-weight compounds possessing a high affinity for iron, which are necessary for the growth of bacteria¹². Some strains of E. coli have several types of siderophores including: A catechol-type siderophore (Enterobactin), a hydroxamate-type siderophore (Aerobactin) and a mixed-type siderophore (yersiniabactin)¹³. Therefore, the present study was proposed to detect virulence factors and genes of E. coli strains isolated from surgical site infections and to evaluate the concomitant presence of plasmids that could be transferred to other species.

**MATERIALS AND METHODS**

**Patients, samples and identification of isolates**

The study comprised (51) strains of E. coli isolated from surgical site infections in patients (one isolate per patient) attending different surgery sections at 5 Academic hospitals in Damascus University.

Samples were collected from 27 females and 24 males aged between 20-60 years with an average age of 40±5 years.

Samples were allocated into the following surgery sections: Maxillofacial and Periodontal Surgery (n= 14), Urological (n= 9), Gastrointestinal (n= 8), Pulmonology (n= 6), Orthopedic Surgery (n= 5), Gynecology (n= 5) and Heart Surgery (n= 4). The study was conducted during the period of (May 2018 – April 2019).

Identification of E. coli strains was done by biochemical tests (API20E, Biomerieux).

**Phenotypic detection of virulence properties of E. coli strains**

- **Determination of 6 virulence factors was performed as follows:**
  - Biofilm formation (BF) was tested by microtiter plate assay procedure¹⁴.
  - Hemagglutination assays for Mannose-sensitive hemagglutination (MSHA) specific for type 1 fimbriae and Mannose-resistant hemagglutination (MRHA) specific for type 3 fimbriae; the procedure was done according to the protocol described by M. Mishra et al. (2001)¹⁵.
  - Hemolysin production (Hly-A) was detected as a clear zone of lysis on blood agar¹⁶.
  - Capsule detection (CPS) by staining with India ink¹⁷.
  - Siderophores production assays were detected by the chrome azurol S (CAS) assay¹⁸.

- **Antibiotic susceptibility testing**

Antibiotic susceptibility was determined by the Kirby-Bauer disk diffusion method on Mueller-Hinton agar. The zones of inhibition were interpreted using the CLSI criteria (CLSI, 2018)¹⁹. The following Antibiotics were tested: Amoxicillin, Cefotaxime, Ceftriaxone, Cefepime, Nitrofurantoin, Doxycycline, Amoxicillin-Clavulanate, Sulfamethoxazole-Trimethoprim, Ciprofloxacin, Levofoxacin, Amikacin, Gentamicin, Imipenem and Colistin.

**DNA and plasmids extraction**

25 of 51 strains had 5 virulence factors (BF, MSHA, MRHA, CPS, Siderophores).

Extraction of DNA and plasmids of these 25 virulent strains was done utilizing
commercially available bacterial genomic DNA Purification kit and Plasmid Purification kit (Intron, Korea). Concentration of extracted genomic DNA and plasmids was measured by Nano drop system (Thermo, USA). DNA and plasmids were stored at (-20°C).

PCR detection of virulence genes

PCR was conducted using specific primers to detect genes encoding type 1 and type 3 adhesins (fimH-1, mrkD), enterobactin biosynthesis (entB), aerobactin receptor (iutA), yersiniabactin biosynthesis (irp-1), Hemolysina (hlyA).

MrkD, iutA and hlyA genes were detected on DNA and plasmids because they could be encoded on both, whereas fimH-1, entB and irp-1 genes were only detected on DNA.

Table 1 demonstrates the Characteristics of the applied PCR protocol.

PCR conditions were: 94°C for 4 min., followed by 30 cycles of 94°C for 30 sec, annealing temperature for 40 sec, 72°C for 1 min, and 72°C for 10 min.16

The electrophoresis of PCR products was performed on 1.5% agarose gels with a proper DNA ladder (Fig. 1). (5 µl) of Ethidium bromide was added to the gel to be visualized with UV system (Cleaver Scientific, UK).

RESULTS AND DISCUSSION

Results

Analysis of virulence factors and antimicrobial resistance

This study was carried out to identify virulence properties of (51) E. coli strains isolated from SSIs. The prevalence of studied virulence factors is shown in figure 2-a. It was observed that (25/51) strains had 5 virulence factors (BF, MRHA, MSHA, CPS, siderophores).

The distribution of virulence factors among (51) E. coli clinical isolates collected from different surgical site infections is shown in figure 3. It was observed that 25 strains showed high virulence (5 virulence factors), 18 showed moderate virulence (3-4 virulence factors) and only 8 were low virulent (1-2 virulence factors).

The evaluation of the susceptibility of the 51 isolates to 14 common antibiotics showed high resistance especially for β-lactam and cephalosporins. The lowest resistance was to colistin, Imipenem and aminoglycosides, (Table 2). Multi drug resistance (MDR) revealed that all isolates were resistant to more than 8 antibiotics and 2 were resistance to all used antibiotics. The 25 strains which had 5 virulence factors showed resistance to more than 11 antibiotics.

Table 1: Primers, annealing temperature and expected size used in study.

| Gene name | Primer sequence (5´–3´) | Annealing temperature (°C) | Expected size (bp) (Amplicon) |
|-----------|-------------------------|---------------------------|-------------------------------|
| fimH-120  | F: ATGAACGCCTGGTCTTTTG  | 55                        | 688                           |
|           | R: GCTGAACGCCTATCCCTGC   |                           |                               |
| mrkD16    | F: CCACCAACTATTCCTCGAA  | 52                        | 240                           |
|           | R: ATGGAAACCAACTCCGACATT|                           |                               |
| entB16    | F: ATTTTCCTAACCCTCGGCG  | 57                        | 371                           |
|           | R: AGCATCGGTGGCGTGGGTCA  |                           |                               |
| iutA21    | F: GGCTGGACACCATGGAGA   | 63                        | 300                           |
|           | R: CGTGGAACGGCTAGAATCG   |                           |                               |
| irp-122   | F: TGAATCGGCTGGTGTTTATGC| 57                        | 238                           |
|           | R: TCCCCCTAAAGAAGGCCAGCT|                           |                               |
| hlyA21    | F: AACAAAAGGATAAGCGACTG | 63                        | 1177                          |
|           | R: ACCATATAAGCGGTCTATTCC|                           |                               |

fim H-1: fimbriae of type 1, mrkD: fimbriae of type 3, entB: enterobactin biosynthesis, iutA: aerobactin receptor, irp-1: yersiniabactin biosynthesis, hlyA: Hemolysin.
Fig. 1: Agarose gel electrophoresis showing amplification products of the *irp1* gene. Lines 1-10: representative the results of amplified product (238bp) of *E. coli* isolates.

Fig. 2: Prevalence of virulence factors and genes of *E. coli* strains.

**Fig (2-a) Prevalence of virulence factors of (51) *E.coli* strains**

- **BF**: Biofilm formation, **MSHA**: Mannose-sensitive hemagglutination, **MRHA**: Mannose-resistant hemagglutination, **CPS**: Capsule, **HLY-A**: Hemolysin

**Fig (2-b) Prevalence of virulence genes on DNA of (25) *E.coli* strains**

- **FimH1**: Fimbriation of type 1, **mrkD**: Fimbriation of type 3, **entB**: Enterobacteriaceae biosynthesis, **lufA**: Aerobacter receptor, **irp1**: Vespivirin activator biosynthesis, **hlyA**: Hemolysin

**Fig (2-c) Prevalence of virulence genes on plasmids of (16) *E.coli* strains**

- **mrkD**: Fimbriation of type 3, **lufA**: Aerobacter receptor, **hlyA**: Hemolysin
Table 2: Antibiotic resistance pattern of (51) *E. coli* strains and antibiogram profiles of MDR (multi drug resistance strains).

| Antibiotic tested               | Resistant strains Number – (%) | MDR                                      | Number (%) |
|---------------------------------|--------------------------------|------------------------------------------|------------|
| Amoxicillin                     | 51 (100%)                      | Resistance to 8 antibiotics              | 7 (13.7%)  |
| Cefotaxime                      | 51 (100%)                      | Resistance to 9 antibiotics              | 10 (19.6%) |
| Ceftriaxone                     | 51 (100%)                      | Resistance to 10 antibiotics             | 9 (17.6%)  |
| Cefepime                        | 49 (96%)                       | Resistance to 11 antibiotics             | 9 (17.6%)  |
| Nitrofurantoin                  | 47 (92.1%)                     | Resistance to 12 antibiotics             | 4 (7.8%)   |
| Doxycycline                     | 43 (84.3%)                     | Resistance to 13 antibiotics             | 10 (19.6%) |
| Amoxicillin-Clavulanate         | 41 (80.4%)                     | Resistance to 14 antibiotics             | 2 (3.9%)   |
| Sulfamethoxazole-Trimethoprim   | 38 (74.5%)                     |                                          |            |
| Ciprofloxacin                   | 35 (68.6%)                     |                                          |            |
| Levofoxacin                     | 33 (64.7%)                     |                                          |            |
| Gentamicin                      | 30 (58.5%)                     |                                          |            |
| Amikacin                        | 28 (54.9%)                     |                                          |            |
| Imipenem                        | 28 (54.9%)                     |                                          |            |
| Colistin                        | 23 (45.1%)                     |                                          |            |

Fig. 3: Distribution of virulence factors of (51) *E. coli* strains according to the surgical site sections.

Fig. 4: Distribution of DNA virulence genes of (51) *E. coli* strains according to the surgical site sections.
Genes study

Extraction protocol of DNA and plasmids involved on (25) strains revealed that plasmids were only found in 16 strains (64%).

Figure (2-b) shows the prevalence of the virulence genes that were detected on DNA. fimH-1 and mrkD genes, encoding type 1 and type 3 fimbrial adhesins, were present in 84% and 96% of isolates respectively. Siderophore genes entB (enterobactin), iutA (aerobactin), irp1 (yersiniabactin) were detected at the prevalences of 92%, 100% and 60%, respectively. Hemolysin A gene (hlyA) was only found in 2 strains.

On the other hand, mrkD, iutA and hlyA genes were also present on the sixteen extracted plasmids (Fig. 2-c). mrkd and iutA were found in 62.5% of plasmids whereas none of them harbored hlyA.

The distribution of virulence genes among (25) virulent E. coli isolates collected from different surgical site infections is shown in (Fig. 4). It was noted that the virulence genes were detected in almost all E. coli strains isolated from General surgery except hlyA which was found only in 2 urological strains, whilst the oral isolates did not show irp1 or hlyA genes.

Discussion

E. coli is considered one of the leading worldwide causes of SSIs. In Syria, the pathogenicity and virulence properties of E. coli in general and especially those isolated from SSIs are not studied enough. In this study we screened E. coli strains for determination of virulence factors, genes and plasmids at selected hospitals in Syria.

Among 51 isolates, more than 70% of them displayed BF, MRHA and MSHA. This may be related to fimbriae role in the first essential step in formation and development of biofilm-associated infections that could shield the bacteria from opsonization and phagocytosis34. Additionally, siderophores was detected in all strains because E. coli is an extracellular pathogen and hence did not have readily access to iron so it produces siderophores to uptake iron from the body. Also, about half of our strains had polysaccharide capsules making them more virulent.

One interesting finding of the present study was that the 25 high virulent strains displayed MDR towards more than 11 antibiotics and 2 against all tested antibiotics. This finding can be attributed to the fact that the hospitalized patients had excessive treatments with antibiotics during their stay in the hospital. The resistance to colistin and Imipenem were lower than other antibiotics due to not being used infrequently and randomly in Syria.

The genes fimH-1 and mrkD were found in a high percentage of our isolates. This result is consistent with the role of type 1 fimbriae as the major factor responsible for the enhanced adhesive and invasive properties of E. coli8, and type 3 fimbriae which allow adhesion to various human tissue structures and are potent promoter of biofilm formation on biotic surfaces35.

According to data from this study entB gene was detected in 23/25 isolates. This finding is in line with previous studies which showed that enterobactin is found in most E. coli strains, both commensal and pathogenic9. Nonetheless, another study pointed to enterobactin's role in the promotion of biofilm development and maturation36.

One remarkable finding of this study was that the iutA gene was present in all isolates, This result suggests the high virulence of the tested strains due to the fact that other studies have reported that the commensal strains do not show this gene25, and the aerobactin receptor shows much greater efficiency in capturing Fe than enterobactin. Paauw et al. has also demonstrated that aerobactin cause indirectly reduced killing capacity of innate immune cells26.

The irp1 gene was found in 15/25 of isolates, thus indicating that each of these 15 strains carries a high-pathogenicity island (HPI) which irp1 gene is encoded on. This result is important in pathogenicity evaluation because (HPI) presence is essential for the expression of a high-virulence phenotype. In addition, it can be considered as an iron-capture island that could spread among various members of the Enterobacteriaceae family by horizontal transfer as suggested by Bach et al.27.

Another notable finding of this study was that the hlyA gene was only present in 2
isolates which were isolated from urology section, this is consistent with other studies that reported that uropathogenic E. coli (UPEC) isolates encode hlyA at high proportion\(^2\).

Plasmids were found in 16/25 strains. Furthermore, 10 of these 16 were virulence plasmids because they possess virulence genes (iutA, mrkD). These plasmids could be transferred to other species contributing to the spread of the aforementioned virulence genes among bacterial populations\(^2\).

**Conclusion**

This study exhibits a high occurrence frequency of some virulence factors (biofilm formation, fimbriae type 1,3 and siderophores) among E. coli strains isolated from SSIs. Moreover, E. coli isolates harbor a high frequency of some virulence genes encoded on genomic DNA (fimH1, mrkD, entB and iutA). In contrast irp1 (encoded on high pathogenicity islands), mrkD and iutA (encoded on virulence plasmids) were detected only in some isolates.

Finally, it could be further presumed that these isolates constitute a genuine threat to the vulnerable populations. Hence, control measures need to be enhanced to prevent these isolates from spreading among Enterobacteriaceae members especially in surgical sections.

**Acknowledgements**

The authors are grateful to Damascus University for funding this research.

**Conflict of interest**

The Authors declare that this study has no conflict of interest.

**Ethical approval**

The study was approved by the Institutional Review Board of Damascus University.

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محددات الفواعة لسلالات الإشريكية القولونية المعزولة من إختيارات المواضع الجراحية من مشفى معينه في سوريا

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تعد الإشريكية القولونية واحدة من أكثر الكائنات المرضية المعزولة من إختيارات المواضع الجراحية والتي تسبب ارتفاعاً هائلاً في معدل انتشار المرض ونسبة الوفيات، وخاصة السلالات ذات الفواعة العالية. لذلك هدفت هذه الدراسة لتقييم محددات الفواعة لسلالات الإشريكية القولونية المعزولة من الأسماك الجراحية. تم دراسة 51 سلالة من الإشريكية القولونية لتفتيح تشكيل البيوفيلم والبتراس الدموي الحساس والمقاوم للمانوز والمخفظة وأنزيم الحالة الدموية وإنتاج حاملات الحديد، كما أجري اختبار التحسس على الصادات الحيوية على 14 صاد حيوي شائع. فحصلنا على 25 سلالة أظهرت عوامل فواعة كما اثبتت مقاومة متعددة لأكثر من 11 صاد حيوي، ثم جرى استخلاص الدنا والبلازميد من السلالات المفوعة (25 ذرية). استخدم فحص البوليميراز التسلسلي PCR لتعري جينات الفواعة (hlyA) والبلازميد الحالة الدموية (fimH-1، mrkD) والبروتينات المحملة علىirim1، iutA، والإبريوبروتينات ، و mrkD على hlyA و iutA. و كانت iutA و mrkD في 21/84% (fimH1) و (23/92%) (entB) و (24/96%) (mrkD) و انتشار متوسط (25/100%) و (2/8) سلالات، و 16/25 (25/100%) و (2/8) سلالات، حيث ظهرت جينات iutA و mrkD و hlyA في الختان، لاحظنا أن معظم السلالات المعزولة من HPI المعزول من إنتشار (25/100%) و (2/8) سلالات، والتي يبدو أنها أساس القدرة الإمبريائية، ولكن بعض السلالات التي تحمل الجزر ذات القدرة الإمبريائية العالية وبايزيمات الفواعة يمكن أن تشكل خطراً حقيقياً إذا انتشرت بين جرامات الإزمنيات الأخرى في الأسماك الجراحية.