Molecular investigation of cytolysin genes among bacterial isolates recovered from pyospermic patients in Hilla City, Iraq

Layth Mousa Hamazah
Biology Department, College of Science, University of Babylon, Hilla City, Iraq

Hussein Oleiwi Muttaleb Al-Dahmoshi
Biology Department, College of Science, University of Babylon, Hilla City, Iraq

*Corresponding author. Email: dr.dahmoshi83@gmail.com

INTRODUCTION

Microbial virulence factors are a group of molecules created by pathogenic bacteria that improve their capability for evading host defenses and causing disease. Toxins, exopolysaccharides, and enzymes are the secreted products that are covered by such a broad definition (Leitão et al., 2020). The main cause of urinary tract infections (UTIs) in humans is Uropathogenic Escherichia coli (UPEC) (Brons et al., 2020). Multiple bacterial toxins, including exotoxins and endotoxins, are secreted by E. coli and difficult to eliminate simultaneously (Jiang et al., 2021). To successfully infect the host and create a favorable environment, UPEC strains require a few unique characteristics, which are achieved through expressing specific genes known as virulence factors. Type-1 fimbriae and P fimbriae are 2 of the major surface virulence factors of UPEC and are essential for urinary tract colonization. When such virulence factors are expressed, a commensal strain becomes a uropathogen. Outer membrane proteins, which are involved in the secretory mechanism, are also contributing to virulence; one example is TolC protein, which transfers -hemolysin over the outer membrane of E. coli. α-hemolysin, like a lot of other toxins, it plays a variety of pathogenic roles in UTI, including colonization, adhesion, cytotoxic activities, and so on (Parvez and Rahman, 2018).

Enterococci are considered important nosocomial bacteria and have many virulence determinants, some of which are significant in pathogenesis. Resistance genes only don't point to the pathogenicity of a bacteria; combined with virulence determinants, it can cause bacteria to become dangerous. Some virulence determinant participates in the colonization, adherence, and evasion of the host's immune responses. The virulence factor genes might be transferred to resistant strains by a competent genetic exchange system (Heidari et al., 2017).

Enzymes, toxins, and exopolysaccharides are examples of secreted products and cell surface structures like lipopolysaccharides, capsules glyco- and lipopro-
teins (Leito, 2020). The toxins are potent pathogenicity factors created by fungi, bacteria, plants, and animals; they also mediate complex interactions between pathogens and their hosts. Bacterial toxins have been the first compounds to be recognized as the cause of serious bacterial infections in animals and humans. (Popoff, 2018). The rapid advancement of whole-genome sequencing of microorganisms will almost certainly lead to the discovery of new toxins and a better understanding of their evolution. Furthermore, refinement of crystal structure investigations allowed for the unravelling of 3-D structures regarding complex or large toxins, like the entire structure of Clostridium difficile's large clostridial glucosylating toxin A (Chumbler et al., 2016). The current study aimed to investigate the cytolyisins among Bacteriospermia associated bacteria in pyospermic patients.

MATERIALS AND METHODS

Collection of the samples
A total of 110 seminal fluid samples from men with primary and secondary infertility disorders that attended the infertility clinic at Babylon Hospital for Maternity and Children and a private laboratory were collected during the period from September 2020 to January 2021., by masturbation, after a 3-day abstinence period. The samples were taken in sterile plastic containers that had previously been used for collecting the samples or urine. Patients washed their genital area and hands with water and soap before collecting the sample.

Culturing and identification
All samples were quickly transferred to the Microbiology Lab, where microbial agents were detected using a standard bacterial culture approach (on chromogenic agar). All isolates were screened via UTI chromogenic agar. The virulence determinant genes for Es. coli: stx1, stx2, stab, sta, lta, hlyA, and sheA (Table 1) and for En. faecalis: hyl, cylLL, and cylLS (Table 2) were identified via PCR by specific primes.

RESULTS AND DISCUSSION

Confirmation of Es. coli and En. faecalis isoaltes performed by PCR using specific primer to amplify uidA and ddl genes for Es. coli and En. faecalis molecular investigation, are mentioned in Fig. 1 and 2 respectively. The results reveal that 30 isolates were E. coli and 30 isoaltes were En. faecalis. The distributions of virulence determinant genes amongst Es. coli and En. faecalis are shown in Fig. 3 and 4. It was found that only one isolate gave a positive result for the gene stx1 1(3.3%), and stx2 gene was found in 6(20%) of Es. coli isolates Fig. 5 and 6. This result was closer to the result of Heydari et al. (2020), who recorded that one isolate (33.30%) carried the stx1 gene and four isolates (66.70%) were positive for stx2 gene of Es. coli, and results of the Rubab and Oh, (2020) who found that 15% and 13% of Es. coli isolates have stx1 and stx2 genes respectively. The frequency of the stx2 gene

Table 1. Primer Sequence and PCR conditions for Es. coli toxins

<table>
<thead>
<tr>
<th>primer</th>
<th>Sequence 5’-3’</th>
<th>Product (bp)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>uida-F</td>
<td>TGGTAATTACGACGAAAACGGC</td>
<td>162</td>
<td>(Bej et al., 1991)</td>
</tr>
<tr>
<td>uida-R</td>
<td>ACGCCGTTGTTACAGTCTTGGC</td>
<td>193</td>
<td>(Han et al., 2007)</td>
</tr>
<tr>
<td>Lta-F</td>
<td>CCCTGCTGACTCTAGACCCCCCA</td>
<td>480</td>
<td>(Kotlowski et al., 2007)</td>
</tr>
<tr>
<td>Lta-R</td>
<td>CCTGCTAATCTGTAACCCTTGTC</td>
<td>278</td>
<td>(Osek et al., 1999)</td>
</tr>
<tr>
<td>Sta-F</td>
<td>ATGAAAGCTAATGTTGGGC</td>
<td>193</td>
<td>(Han et al., 2007)</td>
</tr>
<tr>
<td>Sta-R</td>
<td>TACACAACAGTTGACAGCAG</td>
<td>480</td>
<td>(Kotlowski et al., 2007)</td>
</tr>
<tr>
<td>Stb-F</td>
<td>TGAGAATGGCAAATGTCCG</td>
<td>278</td>
<td>(Osek et al., 1999)</td>
</tr>
<tr>
<td>Stb-R</td>
<td>TGAGAATGGCAAATGTCCG</td>
<td>278</td>
<td>(Osek et al., 1999)</td>
</tr>
<tr>
<td>hlyA-F</td>
<td>GTCTGCAAAAGCAACTCCGCTGCAAATAAA</td>
<td>561</td>
<td>(Kerényi et al., 2005)</td>
</tr>
<tr>
<td>hlyA-R</td>
<td>CTGTGCTCAACAGTTGTTGATTAG</td>
<td>561</td>
<td>(Kerényi et al., 2005)</td>
</tr>
<tr>
<td>sheA-F</td>
<td>GAGGGGAATGATTTATGACTG</td>
<td>920</td>
<td>(Lorenz et al., 2013)</td>
</tr>
<tr>
<td>sheA-R</td>
<td>ATTTGCGGCGTACGTTGGA</td>
<td>920</td>
<td>(Lorenz et al., 2013)</td>
</tr>
<tr>
<td>Stx1-F</td>
<td>GACTCTCAGACTGCAAAGAC</td>
<td>306</td>
<td>(Lorenz et al., 2013)</td>
</tr>
<tr>
<td>Stx1-R</td>
<td>TGATACCAGCTGGTGACCTG</td>
<td>306</td>
<td>(Lorenz et al., 2013)</td>
</tr>
<tr>
<td>Stx2-F</td>
<td>CCCGGAGGTGTACGATAGAC</td>
<td>482</td>
<td>(Lorenz et al., 2013)</td>
</tr>
<tr>
<td>Stx2-R</td>
<td>ACGCAGAACTTGGCTTGATG</td>
<td>482</td>
<td>(Lorenz et al., 2013)</td>
</tr>
</tbody>
</table>
was 15.60% while the frequency of the stx1 toxin gene was 6.30% as reported by (Chandran and Mazumder, 2013). Other studies showed a significantly higher frequency of stx2 but no stx1 compared to this study; the stx2 gene was detected in (66.7%), while stx1 was not found in a study by (Farhan and Al-Iedani, 2019). Gram-negative genera can produce shiga toxin, which prevents protein production in the host cell and results in cell killing. The stx1 and stx2 genes have been considered the most significant virulence factors of the isolated EHEC. The researches showed that the virulence in human beings and animals could be higher when both stx2 than stx1 are present (Heydari et al., 2020). Shiga toxins have been designated as ribosome-inactivating proteins (RIP) are RNA N-glycosidases depurination a particular adenine (A 4324 in rat 28-S rRNAs) so it believed to be Stx1 and Stx2 affect sperm indirectly, causing infertility.

In the Detection of Alpha-hemolysin A (hlyA), it was found that 29(97%) of E. coli isolates have this gene as shown in Fig. 7. These results agreed with the results that have been obtained by Santo and Marin (2006), whose found the ratio of the presence of a gene hlyA in the isolates of Es. coli from urine was (96%). This study disagrees with both (Marrs and Foxman, 2002) and (Düzgün et al., 2019), who found that the ratio of the presence of a hlyA gene were (48% and 6.60%) respectively.

UPEC has been considered the main UTI cause, and they are capable of inducing target uroepithelial cells’ death and exfoliation. Such a procedure may be facilitated by pore-forming toxin alpha-hemolysin (HlyA), secreted and expressed by many isolates of the UPEC. Here, it is demonstrated that the HlyA may result in the possible inhibition of Akt (protein kinase B) activation, one of the key regulators of host cell inflammatory responses, survival, metabolism, and proliferation. The HlyA ablates the activation of the Akt through extracellular K-independent, Ca-dependent process that requires the insertion of the HlyA to the plasma membrane of the host and successive pore formation. The inhibitor studies indicate the fact that the inactivation of the Akt by the HlyA includes the aberrant host protein phosphatase stimulation. The present findings suggest that isolated bacteria can cause UTI and that virulent genes may help these isolates survive even with the use of appropriate antibiotics and are responsible for recurrent infections; local isolates are found to exist relatively at a high level among clinical isolates that have been derived from UTI patients (Wiles et al., 2008). With all of the above, it is believed that this gene enables the bacteria to fulfil its purpose and destroy the sperm by not displaying symptoms by inhibiting the action of the AKT protein, thus exacerbating the infection and obtaining infertility.

STb-STa genes detected by specific PCR primer pairs indicated that all E. coli isolates 30 (100%) have both genes, as shown in (Fig. 8 and 9). The present study showed a considerably higher frequency compared with (Barati, 2012), found that 13.5% and 45.61% harbored STba dnSTa respectively. The gene STa has not been detected in the tested samples (Hassan, 2020) and STb gene preent in 7.41% of isolates. The heat-stable enterotoxins are toxins that are secreted by bacteria Es. coli that LT and STa toxins have been shown lately to result in increased permeability of the epithelial cells that have been observed as trans-epithelial resistance (TER) reduction and passage of the dextran-FITC through the disruption of the TJ. In testes, there’s a blood-testis barrier (BTB) between blood vessels and seminiferous tubules. BTB composition includes interstitial capillary endothelium and the basement membrane, connective tissue, and tight junctions (TJs) between the Sertoli cells (SCs) and the seminiferous epithelium basement membrane. The TJ represents the main structures constituting BTB (Chi et al., 2017). One of the causes of male infertility is the abnormality of the tight junctions in the SCs, which result in spermatogenic cell migration blockage in the seminiferous tubules (Lui et al., 2001). TJ’s barrier function is helpful in the protection of organisms from the pathogens’ entry from the outer environment.
which is why it is usually one of the initial targets for the pathogens, which results in the impairment of the barrier and subsequent processes of inflammation in the status of the disease. Direct pathogen interactions with the tight junction proteins or indirect influences due to their secreted toxins can breakdown the barrier down the intestinal barrier's leading to diarrhea. On the other hand, several pathogenic bacteria do not merely act directly on the tight junctions but also induce apoptosis and, thus, epithelial integrity loss (Krug and Fromm, 2020).

Numerous factors may be responsible for the dysfunctions of the epithelial barrier, which includes microbial infections. The enteric pathogens developed strategies inducing diarrhea production in the infected hosts by disrupting the tight intercellular junctions (Viswanathan et al., 2004). The toxins might be modulating the epithelial barrier through targeting the junctional and cytoskeletal cell components, and thereby, for some pathogens, changes result in the facilitation of invasions over the mucosal surface (Soong et al., 2008). Earlier experimental research has shown a considerable positive association between the increase in intestinal permeability (serum zonulin), sperm DNA oxidative damage, metabolic endotoxaemia (LBP), and increased sperm DNA fragmentation (Halosperm) levels. The metabolic endotoxemia has been positively associated with increased sperm DNA oxidative damage levels, with this correlation staying significant (Pearce et al., 2019).

The \( \text{Ila} \) gene was detected in all \( \text{Es. coli} \) isolates 30 (100%) (Fig. 10). The present study showed a marked higher frequency of \( \text{Ila} \) compared to (Barati, 2012) who found it in 13.5%, whereas no isolates were carrying the \( \text{Ila} \) gene in the study by (Hassan, 2020). Heat-labile toxin (LTa) enterotoxins are secreted by bacteria \( \text{E.coli} \), a well-characterized powerful enterotoxin produced by \( \text{Es. coli} \) (ETEC). It should be noted that it was revealed as well that the LT plays a role in other activities besides its impact on the enterotoxicity. The latest research showed that the LT toxin results in enhancing the enteric adherence of the pathogen and the subsequent intestinal colonizations that LT toxin was recently shown to be causing an increased level of epithelial cell permeability that has been observed as a TER decrease and dextran-FITC passage through the disruption of the tight junctions, which means the fact that it has the same effects as a thermally stable enterotoxin as (STb-STa) (Nassour and Dubreuil, 2014). Therefore, the present study assumes that by affecting the tight junction, the bacteria will fulfill their purpose of destroying the sperm and causing sterility.

Silent hemolysin (\( \text{sheA} \)) gene was detected in 28 (93.3%) of \( \text{Es. coli} \) isolates as shown in (Fig. 11). This result has been similar to the study of (Lorenz et al., 2013) whose found that the \( \text{sheA} \) present in 94%. The present study has shown a considerably higher \( \text{sheA} \) frequency in comparison with Kerényi et al. (2005) whose recorded 47.1% of \( \text{Es. coli} \) isolates recovered from urine had \( \text{sheA} \) and just higher than (Zeb et al., 2021), who found that the \( \text{sheA} \) in frequency in UPEC was 82.8%.\( \text{SheA} \) are toxins secreted by the bacteria damaging the membranes of the cells, which facilitates the infection process. The (ClyA) from \( \text{E. coli} \) is possibly one of the best-characterized examples of the bacterial, a pore–forming toxins (a-PFTs). Similar to other PFTs, the ClyA exists in soluble, monomeric form, assembling to an annular, homo-oligomeric pore complex in the case of contact with a detergent or target membranes (Roderer and Glockshuber, 2017). The carbohydrates play the
role of weak secondary receptors, which are potentially helpful in concentrating the actinoporins on target cell surfaces (Tanaka et al., 2015). Plasma membrane plays the role of semi-permeability barrier between the extracellular environment and the cell, and its integrity is important for the sustainability and survival of the cells. This is why disrupting plasma membrane has been viewed as an ancient cell killing mechanism through which bacteria invade humans. Pore-forming proteins (PFP) are among those molecules which may alter the membrane's permeability (Peraro and Van Der Goot, 2016). Killing target cells by the PFT is one of the most common virulence mechanisms in many different pathogenic bacteria. As largest bacterial toxins' class (Bischofberge et al., 2012). Through the results obtained and from all of the above, the present study believes that toxins perform its purpose by changing the permeability of the membranes and the pores of the sperm and thus damage them and cause infertility. Cytolysins are toxins that are secreted by the bacteria damaging the membranes of the cells, which facilitates the process of the infection can be carried on a plasmid or happen on a bacterial chromosome. The results of the current study indicated that (hyl, cylLL, and cylLS
genes) had the same prevalence and were found in all of the isolates 30(100%) (Fig. 12-14). The present study has shown a considerably higher frequency of cylLL and cylLS compared to Mete et al. (2017); Heidari et al. (2017); Hashem and Aziz (2021); Semedo et al. (2003), who recorded prevalence of 33.2%, 30.4%, 45%, 88%, 2.3% respectively. Precisely the way CylL
" and CylL" sub-units compromise the target cell membranes, which results in the lysis, is not clear yet; however, there is a possibility that it bears a degree of similarity to the pore formation by well-researched lantibiotics nisin and lactacin 3147, both produced by the Lactococcus lactis (Islam et al., 2012). Lantibiotics have shown considerable specificity for some of the components (such as the lipid II) of the membranes of the bacterial cells, particularly of Gram-positive bacteria. Type A lantibiotics are rapidly killed by pore formation; type B lantibiotics inhibit the peptidoglycan biosyntheses (Brötz and Sahl, 2000). Through the present study and from all those who entered the presence of these

Fig. 8. Electrophoresis pattern of PCR product for STb gene. M: Marker 50bp, (PCR product 276bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V, 1h

Fig. 9. Electrophoresis pattern of PCR product for STa gene. M: Marker 50bp, (PCR product 193bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V, 1h

Fig. 10. Electrophoresis pattern of PCR product for LTa gene. M: Marker 100bp, (PCR product 480bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V, 1h

Fig. 11. Electrophoresis pattern of PCR product for sheA gene. M: Marker 50bp, (PCR product 920bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V, 1h
genes that encode for these toxins and that have a cell-analyzing effect, it is believed that they had a destructive effect on the sperm and thus caused sterility.

$hylA$ gene has been detected by specific PCR primer; it was found that (100%) isolates of E. faecalis have this gene, as shown in Fig. 12. This result was closer to the result of Cha et al. (2012) and Shokoohizadeh et al. (2018), whose recorded that the $hylA$ gene was found in (80.3%) of the Enterococcus isolates. Elmalı and Can (2018) and Stępień-Pyśniak et al. (2019) showed that none of the isolates had this gene. Other studies showed a significantly lower frequency of $hyl$ compared to this study; (29.80%) for $hylA$ by Bai et al. (2018) and 50% by Heidari et al. (2017). Gram-positive genera can elaborate that hyaluronidase can cause infections at the skin or mucosal surface of animals or human beings (Hynes et al., 2000).

Typically, the invasion is often facilitated by damage to the host tissues as well as the presence of virulence factors. The bacterial hyaluronidases, enzymes that are able to break down the hyaluronate, are created by several pathogenic Gram-positive bacteria (Hynes et al., 2000). Hyaluronic acid activity (HA) plays a significant role in sperm permeability, motility, and their interactions with the gametes (Bakhtiari et al., 2007). (HA-ICSI) results in considerably improving embryo quality and implantation. When wider multi-center randomized studies confirm those beneficial impacts on the results of the ICSI, hyaluronic acid might be considered one of the routine choices for the "physiologic" sperm selection before the ICSI (Parmegiani et al., 2010). The obtained results have shown the ability of hyaluronidases, enzymes to break down hyaluronate.

**Conclusion**

The present study concludes that both $Es. coli$ and nE. faecalis were more frequent among pyospermia with a set of toxins with possible damage to sperm function, causing indirect infertility like Stx1, Stx2, Stb, Sta, HlyA, SheA and Lta for $Es. coli$ and CyiLL, CyiLS and Hyl for $En. faecalis$.

**Ethical approval**

Informed consent was obtained from all human adult participants, who accepted to culture their semen samples and disposed of them. The project was approved by the Scientific Committee and Bioethics Com
The authors declare that they have no conflict of interest.

REFERENCES


