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Research Article

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

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Abstract
Enterococcus faecalis and Uropathogenic *Escherichia coli* (UPEC) are the most common causal agents of urinary tract infections (UTI) and infertility in humans. They secrete many cytolytic toxins that impact sperm functions and spermatogenesis. The current paper aimed to investigate the cytolysins among Bacteriospermia associated bacteria in pyospermic patients. 110 Seminal fluid swabs were collected from pyospermic patients (age Mean±SD, 35.5±2.12 years) from September 2020 to January 2021. All swabs were inoculated on UTI chromogenic medium for primary isolation of bacteria. Then the suspected *Es. coli* and *En. faecalis* have been confirmed by PCR using *uidA* and *ddl* genes, respectively. The results revealed that *Es. coli* compile 42.9% of bacteriospermia while *En. faecalis* 25.71%. Results of PCR for cytolysins reveal that all *E. coli* isolates have *lta*, *hylA*, *sta*, and *stb* (100%) genes, *sheA* (96.7%), *stx2* (20%), and *stx1*(3.3%). All *En. faecalis* (100%) have Hyl, cylLS, cylLL genes. The current study concludes that both *Es. coli* and *En. faecalis* have a set of toxins with possible damage to sperm function, causing indirect infertility.

Keywords: *Escherichia coli*, *En faecalis*, Bacteriospermia, Pyospermia, Toxins

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 screted 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 *Escherechia 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 favourable 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 cytolysins 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 that had been utilized as a selective medium for isolating the UTI; also, CHROM agar Orientation presents simultaneous presumptive identifications of the Gram-positive and negative bacteria on one medium through the distinct colors of the colony that reactions of the species produced or genus-specific enzymes with a proper chromogenic substrate (Heydari *et al*., 2020; Rubab and Oh, 2020) and was confirmed by the species-specific diagnostic genes of the most frequent isolates.

Virulence factors genes detection using PCR

The virulence determinant genes for *Es. coli*: Stx1, Stx2, Stb, 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 pefromed 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 rspectively. The results reveald 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

primer	Sequence 5′-3′	Product (bp)	Ref.
uidA-F	TGGTAATTACCGACGAAAACGGC	162	(Bej *et al*., 1991)
uidA-R	ACGCGTGGTTACAGTCTTGCG		
Lta-F	CCGTGCTGACTCTAGACCCCCA	480	(Kotlowski *et al*., 2007)
Lta-R	CCTGCTAATCTGTAACCATCCTCTGC		
Sta-F	ATGAAAAAGCTAATGTTGGC	193	(Han *et al*., 2007)
Sta-R	TACAACAAAGTTCACAGCAG		
Stb-F	TGAGAAATGGACAATGTCCG	278	(Osek *et al*., 1999)
Stb-R	TGAGAAATGGACAATGTCCG		
hlyA-F	GTCTGCAAAGCAATCCGCTGCAAATAAA	561	(Kerényi *et al*., 2005)
hlyA-R	CTGTGTCCACGAGTTGGTTGATTAG		
sheA-F	GAGGCGGAATGATTATGACTG	920	(Kerényi *et al*., 2005)
sheA-R	ATTTCGGCGTCACTGTGGAT		
Stx1-F	GACTTCTCGACTGCAAAGAC	306	(Lorenz *et al*., 2013)
Stx1-R	TGTAACCGCTGTTGTACCTG		
Stx2-F	CCCGGGAGTTTACGATAGAC	482	(Lorenz *et al*., 2013)
Stx2-R	ACGCAGAACTTGCTCTGGATG		

Table 2. Primer Sequence and PCR conditions for *En. faecalis* toxins

primer	Sequence 5′-3′	Product (bp)	Ref.
ddl-F	ATCAAGTACAGTTAGTCTT	941	(Comerlato *et al*., 2013)
ddl-R	ACGATTCAAAGCTAACTG		
cylLL-F	GATGGAGGGTAAGAATTATGG	253	(Semedo *et al*., 2003)
cylLL-R	GTATAAGAGGGCTAGTTTCAC		
cylLS-F	GAAGCACAGTGCTAAATAAGG	240	(Semedo *et al*., 2003)
cylLS-R	GTATAAGAGGGCTAGTTTCAC		
Hyl-F	ACAGAAGAGCTGCAGGAAATG	276	(Vankerckhoven *et al*., 2004)
hyl-R	GACTGACGTCCAAGTTTCCAA		

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 TJs. 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 *lta* gene was detected in all *Es. coli* isolates 30 (100%) (Fig. 10). The present study showed a marked higher frequency of *lta* compared to (Barati, 2012) who found it in 13.5%, whereas no isolates were carrying the *lta* gene in the study by (Hassan, 2020). Heat-labile toxin (LTa) enterotoxins are secreted by bacteria E.coli, a well-characterized powerful enterotoxin produced by *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 fulfil their purpose of destroying the sperm and causing sterility.

Silent hemolysin (*sheA*) gene was detected in 28 (93.3%) of *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 *sheA* present in 94%. The present study has shown a considerably higher *sheA* frequency in comparison with Kerényi *et al*. (2005) whose recorded 47.1% of *Es. coli* isolates recovered from urine had *sheA* and just higher than (Zeb *et al*., 2021), who found that the *sheA* in frequency in UPEC was 82.8%.

SheA are toxins secreted by the bacteria damaging the membranes of the cells, which facilitates the infection process. The (ClyA) from 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

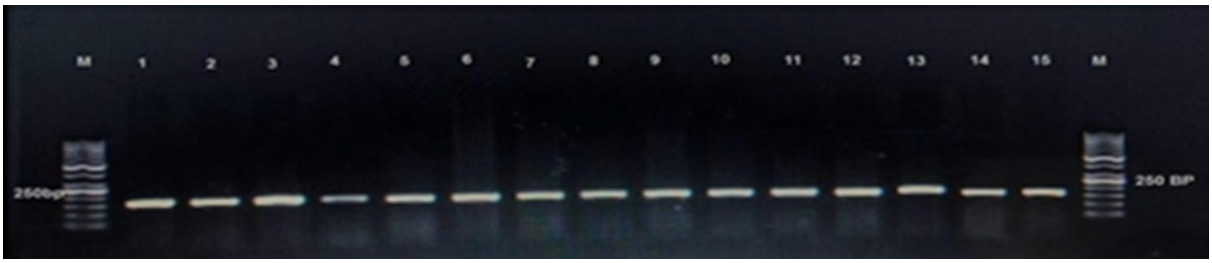

Fig. 1. *Electrophoresis pattern of PCR product for uidA gene. M: Marker 50bp, (PCR product 162bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V,1h.*

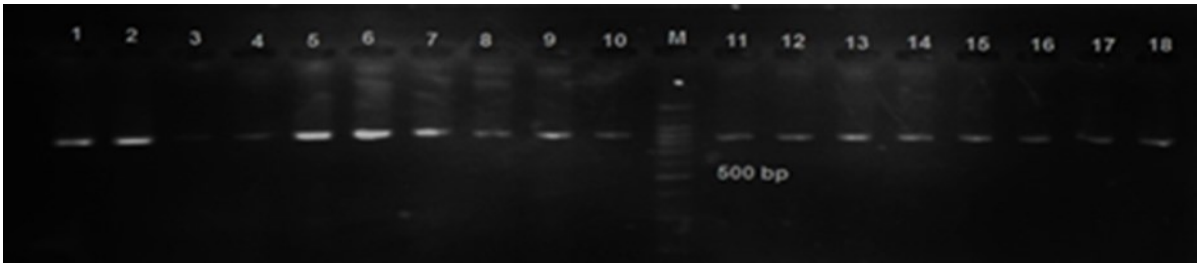

Fig. 2. *Electrophoresis pattern of PCR product for ddl gene. M: Marker 50bp, (PCR product 941bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V,1h*

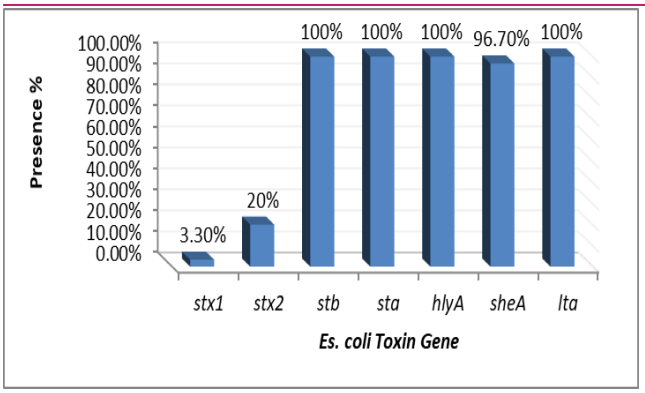

Fig. 3. *Distriburion of toxin genes among Es. coli isolates*

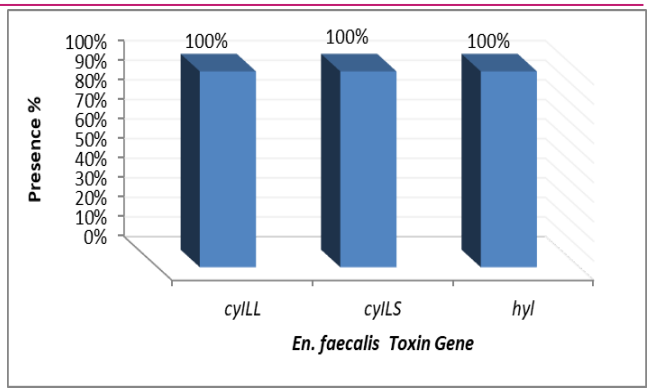

Fig. 4. *Distriburion of toxin genes among En. faecalis isolates*

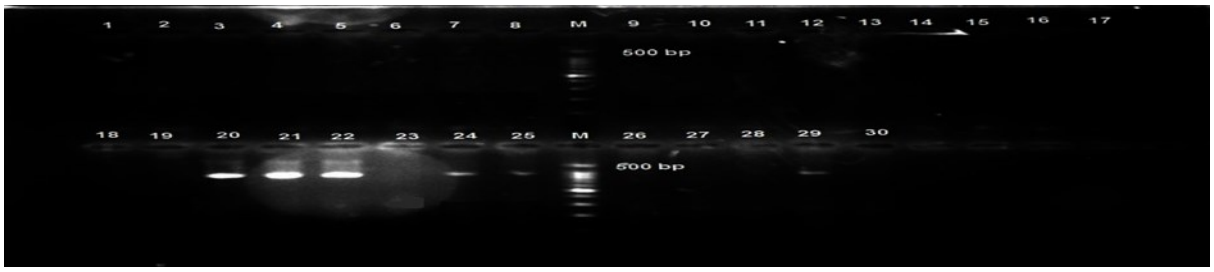

Fig. 5. *Electrophoresis pattern of PCR product for stx2 gene. M: Marker 50bp, (PCR product 480bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V,1h*

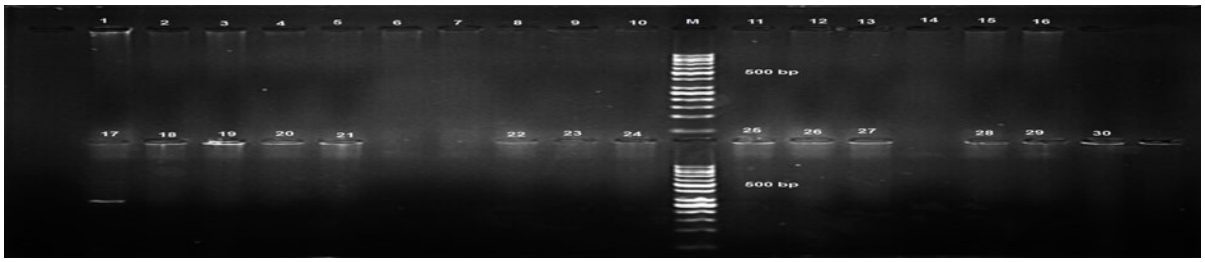

Fig. 6. *Electrophoresis pattern of PCR product for stx1 gene. M: Marker 50bp, (PCR product 306bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V,1h*

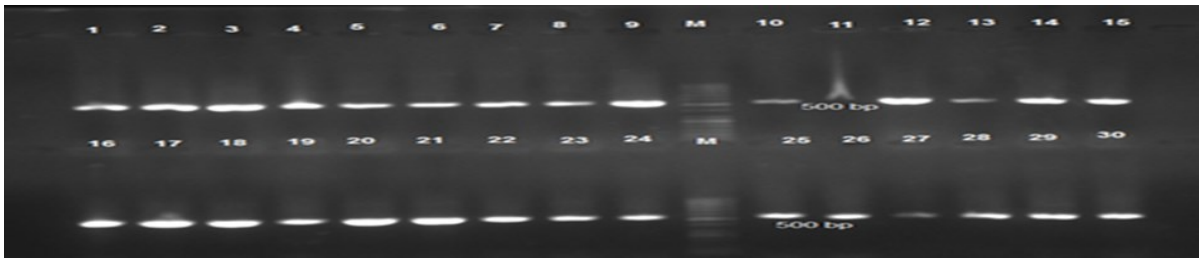

Fig. 7. *Electrophoresis pattern of PCR product for hlyA gene. M: Marker 50bp, (PCR product 561bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V,1h*

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 $CylL_L$" and $CylL_S$" 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 lacticin 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

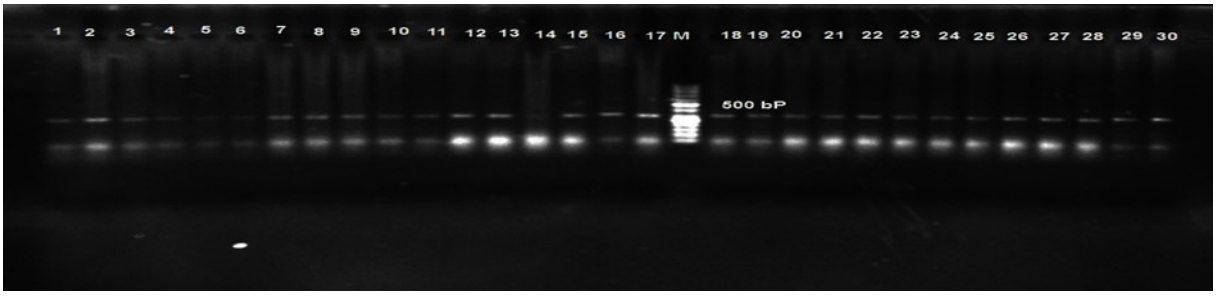

Fig. 8. *Electrophoresis pattern of PCR product for STb gene. M: Marker 50bp, (PCR product 276bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V,1h*

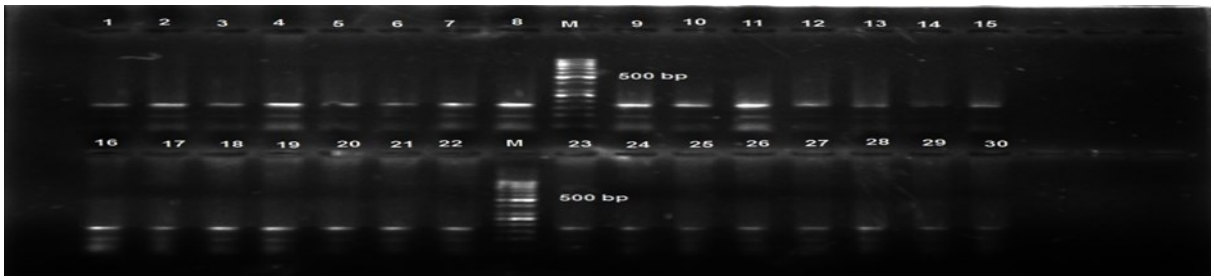

Fig. 9. *Electrophoresis pattern of PCR product for STa gene. M: Marker 50bp, (PCR product 193bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V,1h*

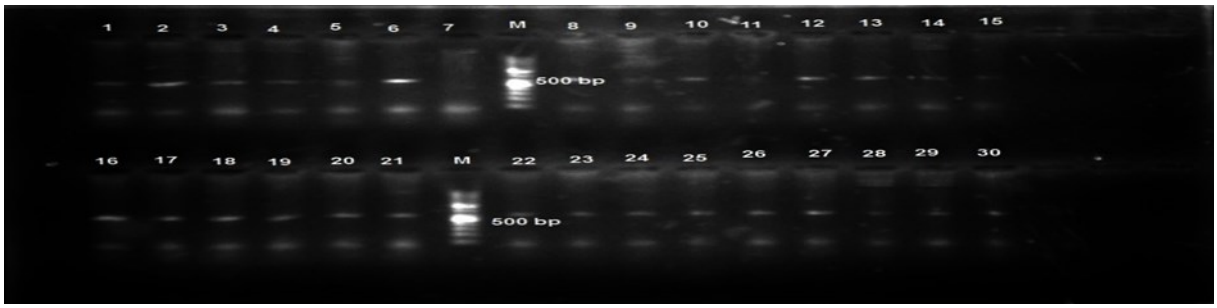

Fig. 10. *Electrophoresis pattern of PCR product for LTa gene. M: Marker 100bp, (PCR product 480bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V,1h*

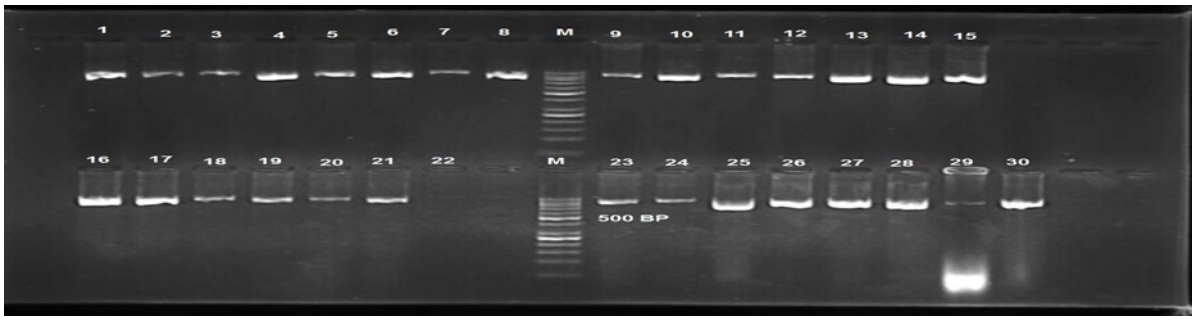

Fig. 11. *Electrophoresis pattern of PCR product for sheA gene. M: Marker 50bp, (PCR product 920bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V,1h*

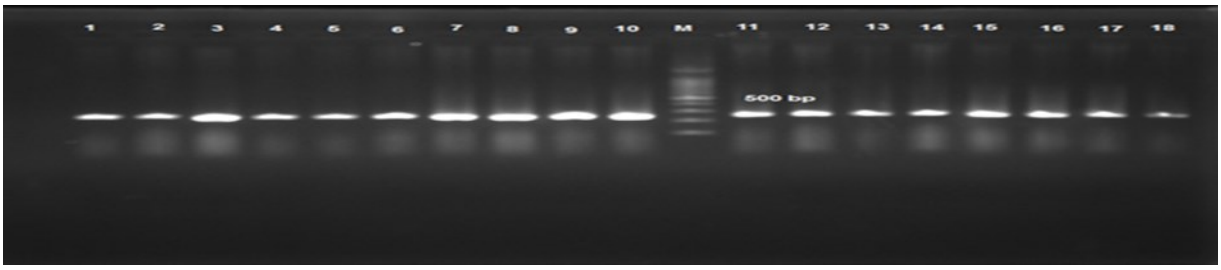

Fig.12. *Electrophoresis pattern of PCR product for cyIL$_L$ gene. M: Marker 100bp, (PCR product 253bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V,1h.*

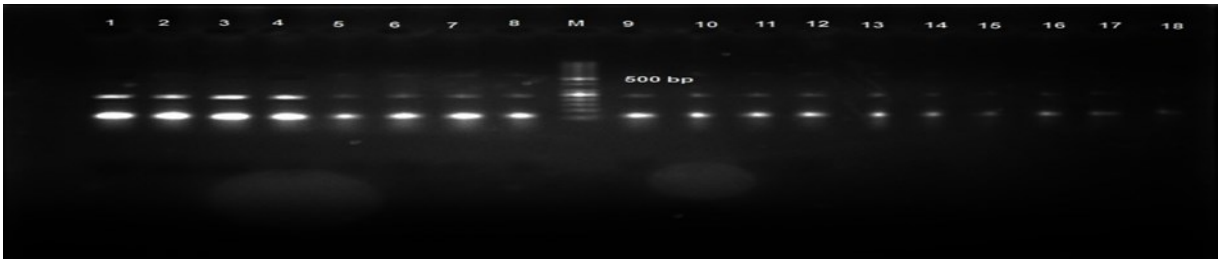

Fig.13. *Electrophoresis pattern of PCR product for cyIL$_S$ gene. M: Marker 50bp, (PCR product 240bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V,1h*

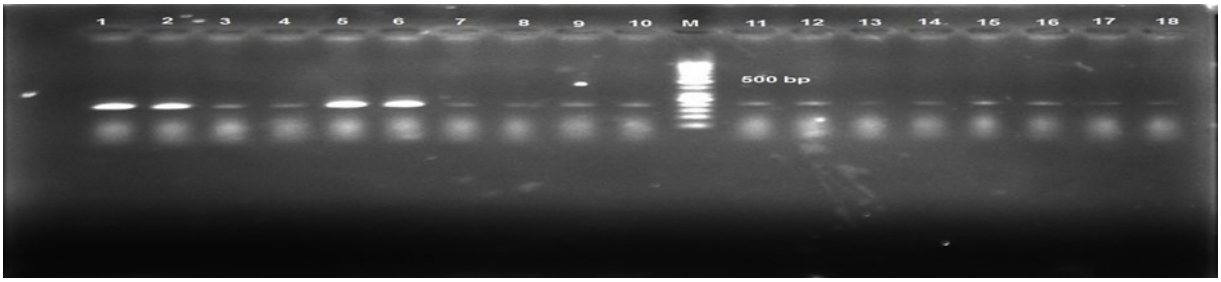

Fig. 14. *Electrophoresis pattern of PCR product for Hyl gene. M: Marker 50bp, (PCR product 276bp) Electrophoresis condition: 2% agarose, TBE 1X 75 V,1h*

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 and Walton,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 n*E. 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-

mittee under project no. M201001 at 7th October 2020. exists.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

1. Bai, B., Hu, K., Li, H., Yao, W., Li, D., Chen, Z., Cheng, H., Zheng, J., Pan, W., Deng, M. & Liu, X. (2018). Effect of tedizolid on clinical *Enterococcus* isolates: in vitro activity, distribution of virulence factor, resistance genes and multilocus sequence typing. *FEMS Microbiology Letters*, *365*(3),1-7.
2. Bakhtiari, M., Sobhani, A., Akbari, M., Pasbakhsh, P., Abasi, M., Hedayatpour, A., Amidi, F. & Sargolzaei, A.F. (2007). The effect of hyaluronic acid on motility, vitality and fertilization capability of mouse sperms after cryopreservation. *International Journal of Reproductive Biomedicine*, 5(2),45-50.
3. Barati, S. (2012). Antibiotic resistance of enterotoxigenic and entroaggrigative *Escherichia coli* isolated from gastroenteritis cases. *Asian Journal of Biomedical and Pharmaceutical Sciences*, 2(14),54-58.
4. Bej, A.K., Dicesare, J.L., Haff, L. & Atlas, R.M. (1991). Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for uid. *Applied and Environmental Microbiology*, *57*(4),1013-1017.
5. Brons, J.K., Vink, S.N., de Vos, M.G., Reuter, S., Dobrindt, U. & van Elsas, J.D. (2020). Fast identification of *Escherichia coli* in urinary tract infections using a virulence gene based PCR approach in a novel thermal cycler. *Journal of Microbiological Methods*, *169*, 1-10
6. Brötz, H. & Sahl, H.G. (2000). New insights into the mechanism of action of lantibiotics—diverse biological effects by binding to the same molecular target. *Journal of Antimicrobial Chemotherapy*, *46*(1),1-6.
7. Cha, J.O., Jung, Y.H., Lee, H.R., Yoo, J.I. & Lee, Y.S. (2012). Comparison of genetic epidemiology of vancomycin-resistant *Enterococcus faecium* isolates from humans and poultry. *Journal of medical microbiology*, *61*(8),1121-1128.
8. Chandran, A. & Mazumder, A. (2013). Prevalence of diarrhea-associated virulence genes and genetic diversity in *Escherichia coli* isolates from fecal material of various animal hosts. *Applied and Environmental Microbiology*, *79* (23),7371-7380.
9. Chi, X., Zhao, X., Wang, W., Niu, Y., Cheng, M., Liu, X., Cui, S. & Yang, W. (2017). Fusion expression of Occludin extracellular loops and an α-helical bundle: A new research model for tight junction. *PloS One*, *12*(4), 1-13.
10. Chumbler, N.M., Farrow, M.A., Lapierre, L.A., Franklin, J.L. & Lacy, D.B. (2016). *Clostridium difficile* toxins TcdA and TcdB cause colonic tissue damage by distinct mechanisms. *Infection and Immunity*, *84*(10), 2871-2877.
11. Comerlato, C.B., Resende, M.C.C.D., Caierão, J. & d'Azevedo, P.A. (2013). Presence of virulence factors in *Enterococcus faecalis* and *Enterococcus faecium* susceptible and resistant to vancomycin. *Memórias do Instituto Oswaldo Cruz*, *108*,590-595.
12. Peraro, M.D. & Van Der Goot, F.G. (2016). Pore-forming toxins: ancient, but never really out of fashion. *Nature Reviews Microbiology*, *14*(2),77-92.
13. Düzgün, A.Ö., Okumuş, F., Saral, A., Çiçek, A.Ç. & Cinemre, S. (2019). Determination of antibiotic resistance genes and virulence factors in *Escherichia coli* isolated from Turkish patients with urinary tract infection. *Revista da Sociedade Brasileira de Medicina Tropical*, *52*,1-5
14. Elmalı, M. & Can, H.Y. (2018). The prevalence, vancomycin resistance and virulence gene profiles of *Enterococcus* species recovered from different foods of animal origin. *Veterinarski Arhiv*, *88*(1),111-124.
15. Farhan, Z.A. & AL-Iedani, A.A. (2019). Molecular detection of shiga toxin (stx1 and stx2) and intimin (eae A) genes in *Escherichia coli* isolated from fecal samples of cattle, sheep, and human in Basrah governorate. *Basrah J Vet Res*, *18*(2),288-305.
16. Han, W., Liu, B., Cao, B., Beutin, L., Krüger, U., Liu, H., Li, Y., Liu, Y., Feng, L. & Wang, L. (2007). DNA microarray-based identification of serogroups and virulence gene patterns of *Escherichia coli* isolates associated with porcine postweaning diarrhea and edema disease. *Applied and Environmental Microbiology*, *73*(12), 4082-4088.
17. Hashem, Y.A., Abdelrahman, K.A. & Aziz, R.K. (2021). Phenotype–genotype correlations and distribution of key virulence factors in *Enterococcus faecalis* isolated from patients with urinary tract infections. *Infection and Drug Resistance*, *14*, p.1713.
18. Hassan, M.H. (2020). The cytotoxicity effects of liable and stable enterotoxins produced by uropathogenic *Escherichia coli*. *Systematic Reviews in Pharmacy*, *11*(12), pp.997-999.
19. Heidari, H., Hasanpour, S., Ebrahim-Saraie, H.S. & Motamedifar, M. (2017). High incidence of virulence factors among clinical *Enterococcus faecalis* isolates in Southwestern Iran. *Infection & Chemotherapy*, *49*(1), pp.51-56.
20. Heydari, F.E., Bonyadian, M., Moshtaghi, H. & Sami, M. (2020). Prevalence and antibiotic resistance profile of Shiga toxin-producing *Escherichia coli* isolated from diarrheal samples. *Iranian Journal of Microbiology*, *12*(4), p.289.
21. Hynes, W.L. & Walton, S.L. (2000). Hyaluronidases of Gram-positive bacteria. *FEMS Microbiology Letters*, *183* (2), pp.201-207.
22. Islam, M.R., Nagao, J.I., Zendo, T. & Sonomoto, K. (2012). Antimicrobial mechanism of lantibiotics. *Biochemical Society Transactions*, *40*(6), 1528-1533.
23. Jiang, L., Zhu, Y., Luan, P., Xu, J., Ru, G., Fu, J. G., Sang, N., Xiong, Y., He, Y., Lin, G. Q., Wang, J., Zhang, J. & Li, R. (2021). Bacteria-anchoring hybrid liposome capable of absorbing multiple toxins for antivirulence therapy of *Escherichia coli* infection. *ACS Nano*, *15*(3), 4173–4185.
24. Kerényi, M., Allison, H. E., Bátai, I., Sonnevend, A., Emödy, L., Plaveczky, N. & Pál, T. (2005). Occurrence of hlyA and sheA genes in extraintestinal *Escherichia coli* strains. *Journal of Clinical Microbiology*, *43*(6), 2965–2968.
25. Kotlowski, R., Bernstein, C. N., Sepehri, S. & Krause, D. O. (2007). High prevalence of *Escherichia coli* belonging

to the B2+ D phylogenetic group in inflammatory bowel disease. *Gut*, *56*(5), 669–675.
26. Krug, S.M. & Fromm, M. (2020). Special issue on "The tight junction and its proteins: more than just a barrier". *International Journal of Molecular Sciences*, *21* (13),4612.
27. Leitão, A. L., Costa, M. C., Gabriel, A. F., & Enguita, F. J. (2020). Interspecies communication in Holobionts by non-coding RNA exchange. *International Journal of Molecular Sciences*, *21*(7), 2333.
28. Lorenz, S. C., Son, I., Maounounen-Laasri, A., Lin, A., Fischer, M., & Kase, J. A. (2013). Prevalence of hemolysin genes and comparison of ehxA subtype patterns in Shiga toxin-producing *Escherichia coli* (STEC) and non-STEC strains from clinical, food, and animal sources. *Applied and Environmental Microbiology*, *79*(20), 6301–6311.
29. Lui, W. Y., Lee, W. M., & Cheng, C. Y. (2001). Transforming growth factor-beta3 perturbs the inter-Sertoli tight junction permeability barrier in vitro possibly mediated via its effects on occludin, zonula occludens-1, and claudin-11. *Endocrinology*, *142*(5), 1865–1877.
30. Marrs, C. F., Zhang, L., Tallman, P., Manning, S. D., Somsel, P., Raz, P., Colodner, R., Jantunen, M. E., Siitonen, A., Saxen, H., & Foxman, B. (2002). Variations in 10 putative uropathogen virulence genes among urinary, faecal and peri-urethral Escherichia coli. *Journal of Medical Microbiology*, 51(2), 138–142.
31. Mete, E., Kaleli, İ., Cevahir, N., Demir, M., Akkaya, Y. & Kiriş Satılmış, Ö. (2017). Evaluation of virulence factors in *Enterococcus* species. *Mikrobiyoloji Bulteni*, *51*(2), 101–114.
32. Nassour, H. & Dubreuil, J.D. (2014). *Escherichia coli* STb enterotoxin dislodges claudin-1 from epithelial tight junctions. *PloS One*, 9(11), 1-11.
33. Osek, J., Gallien, P., Truszczyñski, M., & Protz, D. (1999). The use of polymerase chain reaction for determination of virulence factors of *Escherichia coli* strains isolated from pigs in Poland. *Comparative Immunology, Microbiology and Infectious Diseases*, *22*(3), 163–174.
34. Parmegiani, L., Cognigni, G. E., Ciampaglia, W., Pocognoli, P., Marchi, F. & Filicori, M. (2010). Efficiency of hyaluronic acid (HA) sperm selection. *Journal of Assisted Reproduction and Genetics*, *27*(1), 13–16.
35. Parvez, S.A. & Rahman, D. (2018). Virulence Factors of Uropathogenic *E. coli*. *Microbiology of Urinary Tract Infections-Microbial Agents and Predisposing Factors*,7-21.
36. Pearce, K. L., Hill, A.. & Tremellen, K. P. (2019). Obesity related metabolic endotoxemia is associated with oxidative stress and impaired sperm DNA integrity. *Basic and Clinical Andrology*, *29*(1), 6.
37. Popoff, M. R. (2018). 'Bacterial Toxins' section in the journal toxins: A fantastic multidisciplinary interplay between bacterial pathogenicity mechanisms, physiological processes, genomic evolution, and subsequent development of identification methods, efficient treatment, and prevention of toxigenic bacteria. *Toxins*, *10*(1).
38. Roderer, D. & Glockshuber, R. (2017). Assembly mechanism of the α-pore–forming toxin cytolysin A from *Escherichia coli*. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *372*(1726), 20160211.
39. Rubab, M. & Oh, D. H. (2020). Virulence characteristics and antibiotic resistance profiles of shiga toxin-producing *Escherichia coli* isolates from diverse sources. *Antibiotics*, *9*(9), 587.
40. Santo, E., Macedo, C. & Marin, J. M. (2006). Virulence factors of uropathogenic *Escherichia coli* from a university hospital in Ribeirão Preto, São Paulo, Brazil. *Revista do Instituto de Medicina Tropical de São Paulo*, *48*(4), 185–188.
41. Semedo, T., Almeida Santos, M., Martins, P., Silva Lopes, M. F., Figueiredo Marques, J. J., Tenreiro, R. & Barreto Crespo, M. T. (2003). Comparative study using type strains and clinical and food isolates to examine hemolytic activity and occurrence of the cyl operon in enterococci. *Journal of Clinical Microbiology*, *41*(6), 2569–2576.
42. Shokoohizadeh, L., Ekrami, A., Labibzadeh, M., Ali, L. & Alavi, S. M. (2018). Antimicrobial resistance patterns and virulence factors of enterococci isolates in hospitalized burn patients. *BMC Research Notes*, *11*(1), 1.
43. Soong, G., Parker, D., Magargee, M. & Prince, A. S. (2008). The type III toxins of Pseudomonas aeruginosa disrupt epithelial barrier function. *Journal of Bacteriology*, *190*(8), 2814–2821.
44. Stępień-Pyśniak, D., Hauschild, T., Kosikowska, U., Dec, M. & Urban-Chmiel, R. (2019). Biofilm formation capacity and presence of virulence factors among commensal *Enterococcus* spp. from wild birds. *Scientific Reports*, *9*(1), 11204.
45. Tanaka, K., Caaveiro, J. M., Morante, K., González-Mañas, J. M. & Tsumoto, K. (2015). Structural basis for self-assembly of a cytolytic pore lined by protein and lipid. *Nature Communications*, *6*(1), 6337.
46. Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D. & Goossens, H. (2004). Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium. *Journal of Clinical Microbiology*, *42*(10), 4473–4479.
47. Viswanathan, V. K., Koutsouris, A., Lukic, S., Pilkinton, M., Simonovic, I., Simonovic, M. & Hecht, G. (2004). Comparative analysis of EspF from enteropathogenic and enterohemorrhagic *Escherichia coli* in alteration of epithelial barrier function. *Infection and Immunity*, *72*(6), 3218–3227.
48. Wiles, T. J., Dhakal, B. K., Eto, D. S. & Mulvey, M. A. (2008). Inactivation of host Akt/protein kinase B signaling by bacterial pore-forming toxins. *Molecular Biology of the Cell*, *19*(4), 1427–1438.
49. Zeb, Z., Azam, S., Rehman, N., Khan, I., Afzal, S., Sehra, G. E., Ullah, A. & Absar, M. (2021). Phenotypic and molecular characterization of virulence factors of extra-intestinal pathogenic *Escherichia coli* isolated from patients of Peshawar, Pakistan. *Pakistan Journal of Pharmaceutical Sciences*, 34(1), 85–94.