INTERNATIONAL JOURNAL OF ADVANCED BIOLOGICAL RESEARCH

www.scienceandnature.org

OCCURANCE OF VIRULANCE DETERMENANT IN CLINICAL E.FAECALIS ANDE.FAECIUM ISOLATED FROM URINARY TRACT INFECTIONS IN SOME HOSPITALS IN BAGHDAD REGION

Ashwak B. AL –Hashimy, Aya H. Alhalaby & Hayat Kadhim Salman
*Genetic Engineering and Biotechnology Institute for Postgraduate Studies, Bagdad University.

ABSTRACT
Total of (104) urine samples were collected from patients suffering from urinary tract infection with different age groups from five hospitals in Baghdad (Ibn- Albalady, Al Yarmouk, Medical city, Baghdad hospital and AlKandy) from the period of the beginning of September 2015 to the end of December 2015. All samples were examined by traditional methods based on cultural characteristics, biochemical test and API 20 strep. The results showed the revealed of 50 isolates to Enterococcus and this confirmed by polymerase chain reaction technique based on amplification of species specific genes. PCR were performed for *E. faecalis* and *E. faecium* in order to confirm the presence of Esp which coding for Enterococcus surface protein and EfaA genes which coding for *Enterococcus faecalis* endocarditis antigen using specific primer for each genes, the results showed Enterococcus contain a proportion of 100% of EfaA and 54% for Esp gene.

KEYWORDS: *E. faecalis, E. faecium*, virulence, EfaA, Esp.

INTRODUCTION:

Urinary tract infections (UTIs) comprise one of the largest classes of infections occurring in both hospitals and community (Peleg and Hooper, 2010; Broeren *et al*., 2011). It is estimated that 150 million people with UTIs are diagnosed each year on a global basis, costing in excess of 6 billion dollars in direct health care expenditures (Gupta, 2011), during reproductive life, the urinary tract infections consider important causes of work disabilities and morbidity in general population and is the second most common cause of hospital visits, hence, the need for prophylaxis and prompt treatment (Das *et al*., 2006). Enterococci are the second most common gram-positive urinary pathogen after uropathogenic *E. coli*, also Enterococcus isolated in cases of nosocomial UTIs (Hidron *et al*., 2008; Ortega *et al.*, 2013). Knowledge of the virulence characteristics of circulating Enterococcus strains may help to understand the complex pathogenic process of these opportunistic microorganisms (Sharifi *et al*., 2012). Several Enterococcus virulence factors have been identified, including adhesions and secreted virulence factors. The most important adhesion factors are Asa (aggregation substance), Esp (extracellular surface protein), Efa A (*E. faecalis* antigen A), Ace (adhesion of collagen from *E. faecalis*) and Ebp (endocarditis and biofilm-associated pili) (Fisher and Phillips, 2009). The aim of this study was diagnosis of *E. faecalis* and *E. faecium* from urinary tract infection patients by traditional and molecular methods and detection of Esp and EfaA genes.

MATERIALS & METHODS

Clinical Isolates

Total of (104) urine samples were collected from patients suffering from urinary tract infection with different age groups from five hospitals in Baghdad (Ibn- Albalady, Al Yarmouk, Medical city, Baghdad hospital and AlKandy) from the period of the beginning of September 2015 to the end of December 2015.

Isolation and identification of Enterococcus by traditional methods

Culturing on selective media

The isolates were identified by characteristic colony morphology of Enterococcus on selective media (bile esculin agar) which gave round shape colony with slightly convex smooth edges, creamy color and convert media into black. *Molecular identification of Enterococcus -Bacterial Genomic DNA Extraction Genomic DNA was extracted from the bacterial isolates using Presto Mini g DNA bacteria Kits extraction Genomic DNA, Purification depending on instruction of manufacturing company (Geneaid, Thailand). *Detection of Enterococcus by molecular method-Detection of Enterococcus species by use species specific primer Multiplex PCR used for conformation identification of the *E. faecalis* and *E. faecium*, reaction was conducted in 20 µl of reaction mixture containing 13µl of distilled water, PCR master mix (Bioneer Corporation), 1µl forward from each genes and 1µl reverse primer from each genes, the sequence of primer mention in table (1), finely 3 µl of DNA added (table-2).

TABLE 1: The Sequence of Forward and Reverse Primers used in this study

Genes	Sequence (5' to 3')	Size	Reference
ddl*E. faecium*	F:TTGAGGCAGACCAGATTGACG R:TATGACAGCGACTCCGATTCC	658	Sharifi *et al*.(2012)
ddl *E.faecalis*	F:ATCAAGTACAGTTAGTCTTTATTAG R:ACGATTCAAAGCTAACTGAATCAGT	941	Sharifi *et al*.(2012)

TABLE 2: The Mixture of multiplex PCR working solution for detection of *Enterococcus* species

Component	Volume (µl)
Primer F.	2
Primer R.	2
DNA	3
water	13
Total Volume	20 µl

Amplification was conducted using a DNA thermal cycler programmed with 30 cycles included initial denaturation at 94°C for 10 min, denaturation at 94°C for 1min, annealing at 58°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min as show in table (3), for PCR products were analyzed in agarose gels and visualized under UV after staining with ethidium bromide.

TABLE 3: PCR Program for detection of ddl *E. faecium* and ddl *E. faecalis* genes amplification by multiplex PCR

No.	Steps	Temperature (°C)	Time
1.	Initial Denaturation	94	10 min
2.	Denaturation	94	1 min
3.	Annealing	58	1 min
4.	Extension	72	1 min
5.	Final extension	72	10 min
6.	Cycles number	30	

Detection of Enterococcus virulence genes:

Multiplex PCR were used for detection of virulence genes (Esp and EfaA) in E.faecalis and E.faecium using spesfic primer for each gene. Reaction was conducted in 20 µl of reaction mixture containing 13µl of distilled water, PCR master mix (Bioneer Corporation), 1µl forward from each genes and 1µl reverse primer from each genes, the sequence of primer mention in table (4), finely 3 µl of DNA added (table-5).

TABLE 4: The Sequence of Forward and Reverse Primers for virulence genes used in this study

Genes	Sequence (5' to 3')	Size	Reference
EFaA	F:GACAGACCCTCACGAATA R:AGTTCATCATGCTGTAGTA	705	Eaton and Gasson(2001)
ESP	F:AGATTTCATCTTTGATTCTTGG R:AATTGATTCTTTAGCATCTGG	510	Vankerckhoven *et al*., (2004)

TABLE 5: The Mixture of multiplex PCR working solution for detection of *Enterococcus* virulence genes

Component	Volume (µl)
Primer F.	2
Primer R.	2
DNA	3
water	13

Amplification was conducted using a DNA thermal cycler programmed with 30 cycles included initial denaturation at 95°C for 5 min, denaturation at 94°C for 1 mint, annealing at 56°C at 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min as show in table (6), products were analyzed in agarose gels and visualized under UV after staining with ethidium bromide.

TABLE 6: PCR Program for detection of *Enterococcus* virulence genes amplification by multiplex PCR

No.	Steps	Temperature (°C)	Time
1	Initial Denaturation	95	5 min
2	Denaturation	94	1 min
3	Annealing	56	1 min
4	Extension	72	1 min
5	Final extension	72	10 min
6	Cycles number	30	

RESULTS & DISCUSSION

Clinical Samples

Identification of *Enterococcus* by traditional methods

Fifty isolates identify as *Enterococcus* on bile esculin agar (fig,1) depend on creamy color of colony which conversion of media to black, it consist of 40% bile salt help in inhibition growth of *Streptococci* belong to group D antigen made this media useful in diagnosis of *Enterococcus* from other non-*Enterococcus* bacteria that belong to group D antigen (McFadden, 2000).

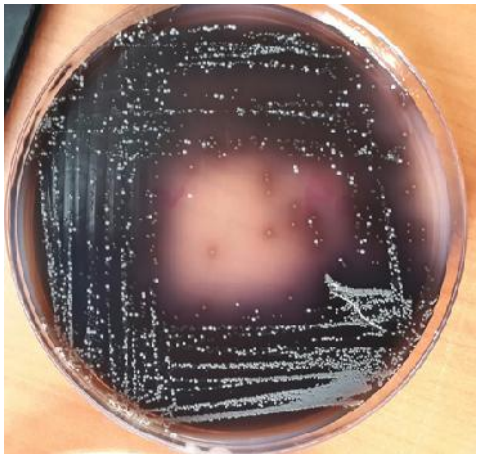

FIGURE 1: Appearance of *Enterococcus* isolates on bile esculin agar

Finally, the API 20 strep system was used for accurate identification of the isolates at generic and species level, the test gave positive results for all isolates as show in fig (2).

FIGURE 2: Biochemical identification of *Enterococcus* using API 20 strep

Identification of *Enterococcus* species by molecular methods

Multiplex PCR technique were used for the diagnosis of all (50) isolates which has grown on the selective media and has already been diagnosed based on their morphology characteristic on culture media and biochemical test, use species-specific primers for the D-alanine-D-alanine ligase gene (ddl *E. faecalis* and ddl *E. faecium*) which was specific for diagnosis of *E. faecalis* and *E. faecium*, it give same result of biochemical test (API 20 strep) 28bacteria isolates for *E. faecalis* and 22 bacteria isolates for *E.faecium*, similar finding was reported by Comerlato *et al*. (2013), piece that amplify by PCR detect by using gel electrophoresis as show in figure (3).

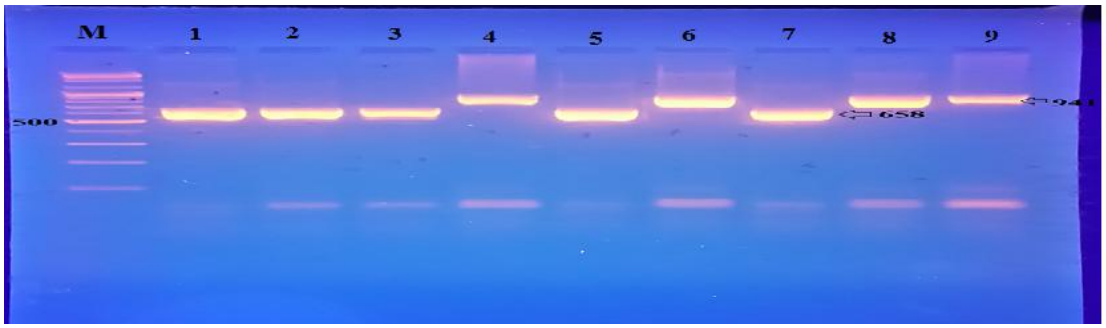

FIGURE 3: Agarose gel electrophoresis of multiplex PCR for identification *Enterococcus* species, M: marker (100pb ladder), lanes (1, 2, 3, 5, 7) positive amplification of ddl *E. faecium* gene (658) pb, lanes (4, 6, 8, 9) positive amplification of ddl *E. faecalis* gene (941) Pb.

Detection of virulence genes in *E.faecalis* and *E.faecium* by multiplex PCR

Several virulence and pathogenicity factors have been described from *Enterococci* that enhance their ability to colonize patient's tissues, increase resistance to antibiotics, and aggravate the infection outcomes (Dupont *et al*., 2008). Multiplex PCR amplification was performed for *E. faecalis* and *E. faecium* in order to confirm the presence of extracellular surface protein (*ESP*) and *E. faecalis* endocarditis antigen (*EFaA*) coding for different virulence factors by use specific primer for each gene , piece of DNA that amplify by multiplex PCR detected by using gel electrophoresis as show in fig. (4).

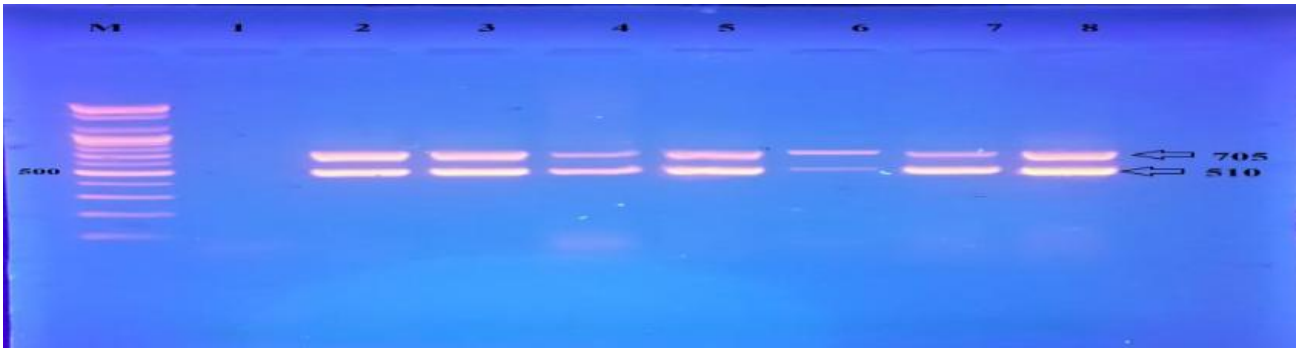

FIGURE 4: Agarose gel electrophoresis of multiplex PCR amplification products of *ESP* (510)pb and *EFaA* (705)pb genes in *E. faecalis* and *E. facium*, M: marker (100 bp ladder) lane 1:negative control, lanes(2-4) :positive amplification of *Esp* and *EFaA* genes in *E. faecalis*, lanes(5-8):positive amplification of *ESP* and *EFaA* genes in *E. faecium*.

The percentage for presence of *ESP* gene in this study for *E. faecalis* were 15 (53.5%), such a low percentage coincide with study by Sharifi *et al*. (2013). While the occurrence of *ESP* genes in *E. faecium* was 12(54.5%), in comparing the current results with other research, it can be concluded that there was similarity with that of Sieńko *et al*. (2015) who observed that biofilm non-producing *E. faecium* harboring *ESP* gene in (55%), while our results disagreement with study of Eaton and Gasson (2001). Furthermore our results show presence *EFaA* gene 100% in *E. faecalis* this finding agree with Cosentino *et al*. (2010) who found that *E. faecalis EFaA* gene presence 100% in urine samples.

The percentage of *EFaA* gene in *E. faecium* was 100%, these results were matched with study of Soheili *et al*. (2014) who found that *EFaA* presence in 100% in *E. faecium*. On the other hand the outcome of this study was disagreement with de Marques and Suzart (2004).We chose to detect Esp and EfaA genes for their importance in Enterococcus pathogenicity as Esp contribute to colonization and persistence of *E. faecalis* in ascending infections of urinary tract also Esp participate in biofilm formation which enhance bacterial survival in biopolymers and also involve in antimicrobial resistance(Chuang-Smith *et al*., 2010; Ballering *et al*., 2009). EfaA gene involve in adhesion of Enterococcus to biotic and abiotic surface or evasion of immune response (Pérez-Pulido *et al*., 2006).

REFERENCES

Peleg, A.Y. and Hooper, D.C. (2010) Hospitalacquired infections due to gram-negative bacteria. New England Journal of Medicine, 362(19): 1804-1813.

Broeren, M.A., Bahçeci, S., Vader, H.L. and Arents, N.L. (2011) Screening for urinary tract infection with the Sysmex UF-1000i urine flow cytometer. Journal of clinical microbiology, 49(3): 1025-1029.

Gupta, K., Hooton, T.M., Naber, K.G., Wullt, B., Colgan, R., Miller, L.G. and Soper, D.E. (2011) International clinical practice guidelines for the treatment of acute uncomplicated cystitis and pyelonephritis in women: a 2010 update by the Infectious Diseases Society of America and

the European Society for Microbiology and Infectious Diseases. Clinical infectious diseases, 52(5): e103-e120.

Das, R.N., Chandrashekhar, T.S., Joshi, H.S., Gurung, M., Shrestha, N. and Shivananda, P.G. (2006) Frequency and susceptibility profile of pathogens causing urinary tract infections at a tertiary care hospital in western Nepal. Singapore medical journal, 47(4): 281.

Hidron, A.I., Edwards, J.R., Patel, J., Horan, T.C., Sievert, D.M., Pollock, D.A. and Fridkin, S.K. (2008) National Healthcare Safety Network Team; Participating National Healthcare Safety Network Facilities. NHSN annual update: antimicrobialresistant pathogens associated with healthcareassociated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infect Control Hosp Epidemiol, 29(11): 996-1011.

Ortega, M., Marco, F., Soriano, A., Almela, M., Martinez, J.A., Pitart, C. and Mensa, J. (2013) Epidemiology and prognostic determinants of bacteraemic catheter-acquired urinary tract infection in a single institution from 1991 to 2010. Journal of Infection, 67(4): 282-287.

Sharifi, Y., Hasani, A., Ghotaslou, R., Varshochi, M., Hasani, A., Aghazadeh, M. and Milani, M. (2012) Survey of virulence determinants among vancomycin resistant Enterococcus faecalis and Enterococcus faecium isolated from clinical specimens of hospitalized patients of North west of Iran. The open microbiology journal, 6(1).

Fisher, K. and Phillips, C. (2009) The ecology, epidemiology and virulence of Enterococcus. Microbiology, 155(6): 1749-1757.

Eaton, T.J. and Gasson, M. J. (2001) Molecular screening of Enterococcus virulence determinants and potential for genetic exchange between food andmedical isolates. Applied and environmental microbiology, 67(4): 1628-1635.

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R. and 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.

MacFaddin, J.F. (2000) Biochemical tests for identification of medical bacteria, 3rd ed. Lippincott Williams and Wilkins, Baltimore.

Comerlato, C.B., Resende, M.C.C.D., Caierão, J., and 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(5): 590-595.

Dupont, H., Vael, C., Muller, C., Chosidow, D., Mantz, J., (2008) Prospective evaluation of virulence factors of Enterococci isolated from patients with peritonitis: impact on outcome.Diag. Microbiol. Infect. Dis. 2008; 60(3): 247-53.

Sieńko, A., Wieczorek, P., Majewski, P., Ojdana, D., Wieczorek, A., Olszańska, D. and Tryniszewska, E. (2015) Comparison of antibiotic resistance and virulence between biofilm-producing and nonproducing clinical isolates of *Enterococcus faecium*. Acta Biochimica Polonica, 62(4): 859-866.

Cosentino, S., Podda, G.S., Corda, A., Fadda, M.E., Deplano, M., and Pisano, M.B. (2010) Molecular detection of virulence factors and antibiotic resistance pattern in clinical *Enterococcus faecalis* strains in Sardinia. Journal of preventive medicine and hygiene, 51(1). 16. Soheili, S., Ghafourian, S., Sekawi, Z., Neela, V., Sadeghifard, N., Ramli, R. and Hamat, R.A. (2014) Wide distribution of virulence genes among *Enterococcus faecium* and *Enterococcus faecalis* clinical isolates. The Scientific World Journal, 2014.

De Marques, E.B. and Suzart, S. (2004) Occurrence of virulence-associated genes in clinical Enterococcus faecalis strains isolated in Londrina, Brazil.Journal of medical microbiology, 53(11): 1069-1073.

Chuang-Smith, O.N., Wells, C.L., Henry-Stanley, M.J. & Dunny, G.M. (2010) Acceleration of Enterococcus faecalis biofilm formation by aggregation substance expression in an ex vivo model of cardiac valve colonization. PLoS One, 5(12): e15798.

Ballering, K.S., Kristich, C.J., Grindle, S.M., Oromendia, A., Beattie, D.T. & Dunny, G.M. (2009) Functional genomics of *Enterococcus faecalis*: multiple novel genetic determinants for biofilm formation in the core genome. Journal of bacteriology, 191(8): 2806-2814.

Pérez-Pulido, R., Abriouel, H., Omar, N. B., Lucas, R., Martinez-Canamero, M. & Galvez, A. (2006) Safety and potential risks of Enterococci isolated from traditional fermented capers. Food and chemical toxicology, 44(12): 2070-2077.