ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Molecular Detection for Virulence Factors of Coagulase Negative Staphylococci Isolated from Patients with Urinary Tract Infection.

Mohammed Sabri Abdul-Razzaq*, Jawad Kadhim Al-Khafaji, and Sarah Hashim Al-Helli.

University of Babylon, College of Medicine, Department of Microbiology, Hilla-Iraq.

ABSTRACT

Coagulase Negative Staphylococci (CoNS), are typical opportunists, represent one of the major nosocomial pathogens, having a substantial impact on human life and health. This study aimed to genetically investigate some virulence factors of CoNS isolated from patients with UTI. This study included 120 urine sample were obtained from patients suffering from UTI and subjected to culture to identify CoNS that followed by DNA extraction and PCR technique. CoNS are Gram +ve cocci, that were detected in 37/110 +ve culture. Coagulase gene (*coa*) was positive in 75.67% of CoNS in all *S. epidermidis*, *S. saprophyticus* and *S.haemolyticus* distributed as (15, 9 and 4) respectively. Also *fnb* A and B genes were detected in most cases, while, all isolates are free from *sec* gene. Molecular technique for detection of virulence gene in CoNS display that these bacteria may have *coa* gene in silent state.

Keywords: CoNS, Gram-positive bacteria, PCR, UTI.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Staphylococci is entirely gram-positive, non-motile, not producing spores ubiquitous bacteria that include different opportunistic/pathogenic species, responsible for human and animal infections [1]. Depending on coagulase production, *Staphylococcus* species are classified into two type; coagulase-positive staphylococci (CoPS) that include *Staphylococcus aureus* specie and Coagulase-Negative Staphylococci (CoNS); as the later one are particularly associated with the use of indwelling or implanted foreign bodies, which are indispensable in modern medicine. Colonization of the skin and mucous membranes of the host is the key source of endogenous infections by CoNS [2]. The main staphylococcal species of clinical interest could be identified by the coagulase test and Novobiocin susceptibility that *S. aureus* is coagulase positive and Novobiocin sensitive; *S. epidermidis* and *S. haemolyticus* are coagulase negative and Novobiocin sensitive; *S. saprophyticus*: coagulase negative and Novobiocin resistant [3].

Lisa [4] and Vianello [5] mentioned that there are thirty four different extracellular proteins are produced by pathogenic *Staphylococcus* strains, and several of them are already play a specific role in the pathogenesis of recognized staphylococcal disease. On the basis of the study done by Peacock *et al*. [6], the number of virulence- associated genes carried by a bacterial strain is the product of interaction between the gene's acquisition rates, the cost for biological maintenance and the failure rate of the strain causing the disease.

Coagulase is an enzyme produced by several bacteria that enables the conversion of fibrinogen to fibrin, which protects the bacterium from phagocytosis and isolate it from other defenses of the host. It has been proposed that fibrin-coated staphylococci resist phagocytosis, making the bacteria more virulent [7].

Fibronectin-binding protein A (FnBPA) and Fibronectin-binding protein B (FnBPB) are specific adhesins for *Staphylococcus* infections. FnBPA is necessary for *in vitro* and *in vivo* infections. So, the cooperation between FnBPA and FnBPB is very essential for the induction of severe diseases [8].

There are two genes encoding for fibronectin-binding proteins which have been identified in *S. aureus* *fnb*A and *fnb*B. Fibronectin binding activity is critical in pathogenesis because it allows the bacteria to adhere to extracellular matrix components including fibronectin and also collagen. This can result in cutaneous infections and in life-threatening bacteremia and endocarditis [9].

This study aimed to investigate some virulence factors of CoNS, genetically.

MATERIALS AND METHODS

A total of 120 urine sample (100 female samples and 20 male samples) were obtained from patients suffering from urinary tract infection; who attained to Babylon Maternity and Pediatrics Hospital, and Al-Hilla General Teaching Hospital, during the period from December 2015 to March 2016, they were diagnosed as having UTI by the Urologists. The samples were analyzed for any signs of infection and immediately inoculated on blood, MacConkey and Mannitol-salt agar plates. All plates were incubated aerobically at 37 ºC for 24-48 hrs. Coagulase test done for all suspected *Staphylococcus* spp. and then the coagulase negative Staphylococci tested with Novobiocin disk. Later on all CoNS positive cultures were subjected to DNA extraction using Geneaid/UK Kit to be utilized in the amplification of virulence factors genes using specific primers with sequences and amplification conditions as listed in table (1). Followed by detection of the amplicons using UV transilluminator.

ISSN: 0975-8585

Table (1): Sequences of primers and their PCR conditions.

Genes	Primer sequence	Size of product bp	PCR condition	Reference
sec F *sec* R	5'GACATAAAAGCTAGGAATTT3' 5'AAATCGGATTAACATTATCC3'	257 bp	94°C 1min 92°C 45min 46°C 45min } 30X 72°C 45min 72°C 10min	[10]
coa F *coa* R	5'GTA GAT TGG GCA ATT ACA TTT TGG AGG3' 5'CGC ATC AGC TTT GTT ATC CCA TGT A3'	117 bp	94°C 1min 92°C 45min 55°C 1min } 30X 72°C 45min 72°C 10min	[11]
fnbA F *fnbA* R	5'GCGGAGATCAAAGACAA3' 5'CCATCTATAGCTGTGTGG3'	1278bp	94°C 1min 92°C 1min 50°C 1min } 30X 72°C 1min 72°C 10min	[10]
fnbB F *fnbB R*	5'GGAGAAGGAATTAAGGCG3' 5'GCCGTCGCCTTGAGCGT3'	812 bp	94°C 1min 92°C 1min 50°C 1min } 30X 72°C 1min 72°C 10min	[10]

Ethical Approval:

A valid consent was achieved from each patient before their inclusion in the study. For every patient, the procedure had been informed before the samples were collected, making absolutely sure that they understood the procedure that was to be carried out. The subjects were sentient that they had the right to reject to be included in the study without any detrimental effects.

RESULTS AND DISCUSSION

Among 120 clinical samples only 110 showed positive results, among them 37 isolates are belonged to CoNS, other 73 isolates belong to other bacterial genera as shown in table (2).

Table (2): Number and percentage of bacteria isolated from urine samples.

No. of urine samples	No. of positive culture		No. of Negative culture
	No. of CoNS	No. of other bacteria	
120 samples	37(31%)	73(61%)	10(8%)

Coagulase negative Staphylococci that were isolated and identified in this study, are mostly belonging to the *S. epidermidis* (17 isolates), *S. saprophyticus* (12 isolates), and *S. haemolyticus* (8 isolates), as shown in figure (1).

ISSN: 0975-8585

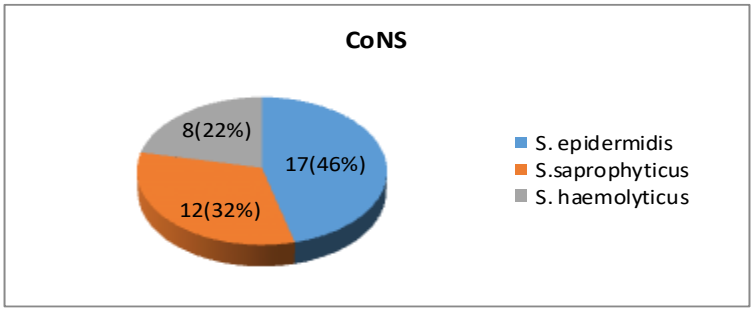

Figure (1): No. of Staphylococcal species among CoNS isolates.

The identification of CoNS depends mainly on the cultural, biochemical characteristics and microscopic patterns. These organisms are grams-positive cocci microscopically appear as grape-like clusters, non-motile , non-spore forming, coagulase negative, catalase positive and oxidase negative [12]. Most *Staphylococcus*, on blood agar, the colonies tend to be nonpigmented, smooth, entire, glistening, and opaque colonies. Indeed, *S. saprophyticus* on Mueller-Hinton Agar medium exhibiting resistance to Novobiocin, whereas *S. epidermidis* and *S. haemolyticus* are Novobiocin-sensitive. However, *S. epidermidis* showed resistance to PolymyxinB, whereas *S. saprophyticusand S. haemolyticus* are PolymyxinB-sensitive. These findings have been noted in similar studies done on CoNS of which Sarathbabu *et al*., [13].

Molecular detection of coagulase gene (*coa*) was done for all CoNS isolates, with an amplicon size at 117bp when compared with allelic ladder as shown at figure (2). The results showed that 28 isolates (75.67%) gave positive result for this marker where *S. epidermidis* gave 15 isolates, *S. saprophyticus* gave 9 isolates and *S. haemolyticus* gave 4 isolates, as shown in table (3).

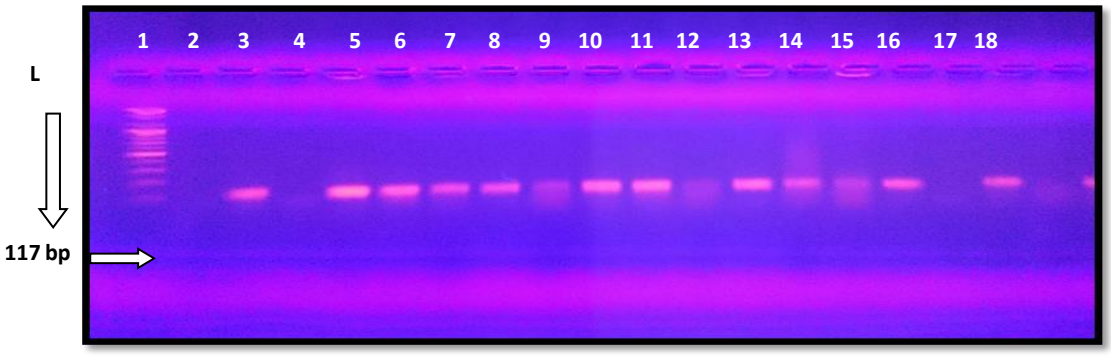

Figure (2): 1.5% Agarose gel electrophoresis at 70 volt for 50 min for *coa* PCR products visualized under U.V light at 280 nm after staining with ethidium bromide. L: 1500 bp ladder; lane (2,4,5,6,7,8,9,10,11,12,13,14,15,17) were positive for this gene, CoNS in urine samples among patients with UTI. The size of product is 117 bp.

Table (3): Distribution of virulence factor genes among CoNS isolates

Genes	No. of isolate			Total
	S.saprophyticus	*S.haemolyticus*	*S.epidermidis*	
coa	9	4	15	28(75.67%)
*fnb*A	5	2	6	13(35.1%)
*fnb*B	2	0	2	4(10.8%)
sec	0	0	0	0(0%)

Although these bacteria are negative for coagulase test, but the presence of *coa* gene may indicate that there is sequence homology to the *S. aureus* gene but there is no transcriptional state for this gene or there is no specific secretion system to secrete this enzyme extracellularly or the gene inside CoNS remains silenced with no function.

ISSN: 0975-8585

However, some isolates gave negative results for this marker, this means that *coa* gene in these isolates is either mutated or absent. This result is similar to that obtained by Moura *et al*, [14] who observed that although all CoNS strains are negative for coagulase test, but more than 75% are positive for *coa* gene. Moreover, Mulu *et al*., [15] have stated down that coagulase test when be negative in CoNS bacteria mean that these bacteria did not *S. aureus* in the presence of *coa* gene, where the gene may be present as a result of gene transfer, recombination, DNA integration and DNA rearrangement.

Regarding molecular investigation of *fnb* A and B genes are done for all CoNS isolates by using specific PCR markers, with results at table (3). Thirteen isolates (35%) gave positive results for *fnb* A gene, where six isolates of *S. epidermidis*, five isolates of *S. saprophyticus* and two isolates of *S. haemolyticus* have this gene in their genome as shown in figure 3, with an amplicon size at 1278 bp when compared with allelic ladder.

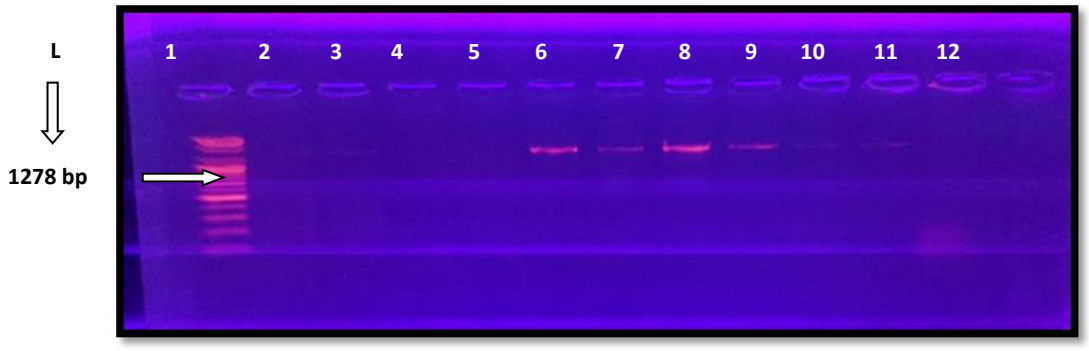

Figure (3): 1.5% Agarose gel electrophoresis at 70 volt for 50 min for *fnb A* PCR products visualized under U.V light at 280 nm after staining with ethidium bromide. L: 1500 bp ladder; lane (5,6,7,8,9,10) were positive for this gene, CoNS in urine samples among patients with UTI. The size of product is 1278 bp.

On the other hand, only four isolates gave positive results for *fnb* B gene where two isolates of each *S. saprophyticus* and *S. epidermidis* have this gene in their genome as shown in figure (4).

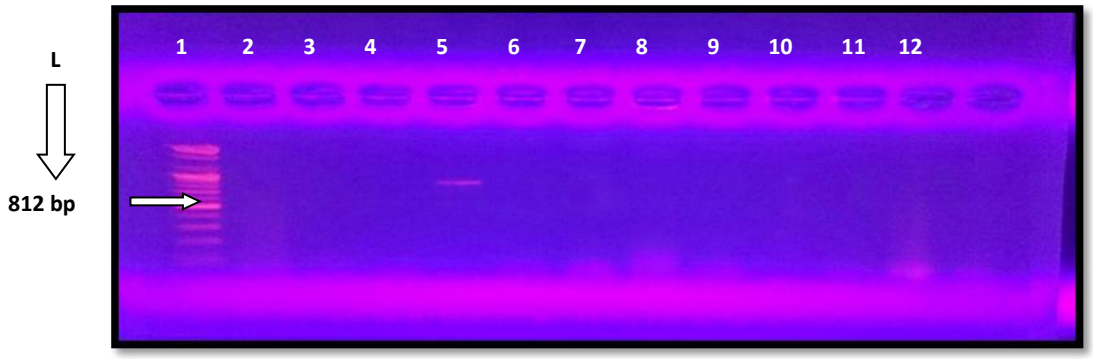

Figure (4): 5% Agarose gel electrophoresis at 70 volt for 50 min for *fnb B* PCR products visualized under U.V light at 280 nm after staining with ethidium bromide. L: 1500 bp ladder; lane (4) were positive for this gene, CoNS in urine samples among patients with UTI. The size of product is 812 bp.

Both *fnb* A and B have been demonstrated to be present in *S. aureus* and they have a role in bacterial adhesion and pathogenesis [16].However, the presence of each genes in CoNS may increase their adhesion in different tissues and also in internalization of the microbes to the cells [17].According to the data obtained in this study, it was observed that both *fnb* A and B are seen only in *S. saprophyticus* but not in other CoNS isolates. So the present of both *fnb* A and B in *S.saprophyticus* may increase the pathogenecity of this bacteria because the functions of *fnb* A and B proteins are cooperative *in vivo* and *in vitro* infections [18]. So it is worthy conclude that *S.saprophyticus* is true human pathogens where it has many functional proteins that may have a role in infection and invasion.

ISSN: 0975-8585

Finally, *sec* gene was investigated in CoNS isolates, and results showed that all CoNS isolates are free from this locus as shown in figure (5) and table (3), it means that there is no sequence homology or gene identical to *S. aureus sec* gene.

However, many studies indicated the same result that *S. epidermidis* does not confirm its ability to produce enterotoxins [19, 20]. It was indicated the ability of *S. haemolyticus* to produce at least only are one type of enterotoxines that are known in *S. aureus* [20, 21], and cytolysin production is the main virulence factor in this bacteria particularly when it reaches the blood stream [21, 22, 23].

However, the results of this study confirm the absence of staphylococcal enterotoxin gene within CoNS isolates and this means that these isolates are not enterotoxigenic and not virulent when present in human intestine accidently, and this is in contrast with that obtained by Veras *et al*.[24] who found that many CoNS are enterotoxigenic. On other hand, *S. saprophyticus* isolates are also freed from this locus and there was no previous evidence on the ability of this bacteria to cause food poisoning [25].

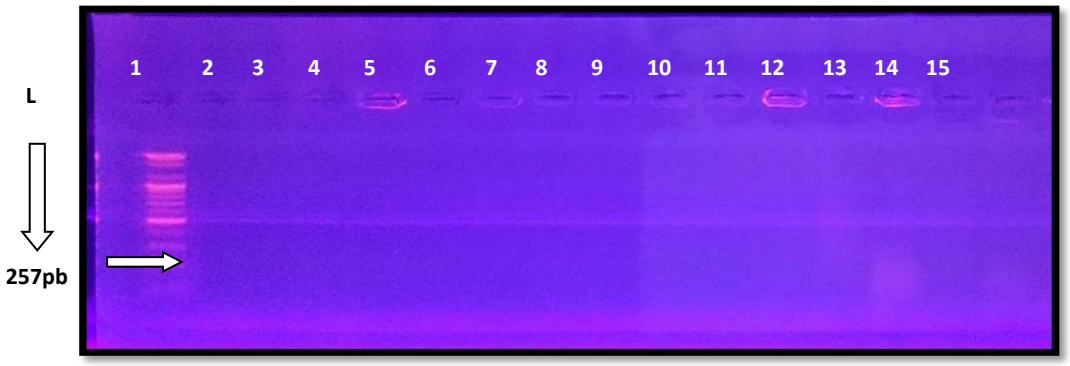

Figure (5): 1.5% Agarose gel electrophoresis at 70 volt for 50 min for *sec* PCR products visualized under U.V light at 280 nm after staining with ethidium bromide. L: 1500 bp ladder; No positive for this gene, CoNS in urine samples among patients w ith UTI. The size of product that should appear is 257 bp.

ACKNOWLEDGMENT

The authors are thankful to Department of Microbiology, College of Medicine, Babylon University for their facilities in the completion of the work. Also thankful to the patients for their cooperation. I am also indebted to the staff of microbiology laboratory in Babylon Maternity and Pediatrics Hospital, and Al-Hilla General Teaching Hospital for all the facilities they provided to me .

REFERENCES

[1] Makris, G., Wright, J. D., Ingham, E. and Holland, K. T. (2004). The hyaluronate lyase of *Staphylococcus aureus* – a virulence factor? Microbiology, Vol.150, 2005-2013.

[2] Pyorala, S. and Taponen, S. (2009). Coagulase-negative staphylococci-emerging mastitis pathogens. Vet. Microbiol., 16(134):3-8.

[3] Ferreira, A. et al. (2012). Identification of *Staphylococcus saprophyticus* isolated from patients with urinary tract infection using a simple set of biochemical tests correlating with 16S–23S interspace region molecular weight patterns. J Microbiol Meth. 91: 406–411.

[4] Lisa, R. (2004). *Staphylococcus aureus* exfoliative toxins: How they cause disease. Derm. Foundat. 122: 1070-1077.

[5] Vianello, M. (2006). Caracterizacao genotipica dos fatores de virulencia e seu regulador *agr* em cepas de *Staphylococcus aureus* sensiveis a oxacilina.

[6] Peacock, S., Moore, C., Justice, A., Kantzanou, M., Story, L., Mackie, K., O'Neill, G. and Day, N. (2002). Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. Infect Immun. 70(9): 4987-4996.

[7] Tortora, G., Funke, B. and Case, C. (2013). Microbiology: An Introduction (11th. edn.). Glenview, IL: Pearson Education Inc. 434.

[8] Shinji, H., Yosizawa, Y., Tajima, A., Iwase, T., Sugimoto, S., Seki, K. and Mizunoe, Y. (2011). Role of fibronectin-binding proteins A and B in in vitro cellular infections and in vivo septic infections by *Staphylococcus aureus*. Infect Immun. 79(6): 2215-2223.

[9] Al-Hasseny, R. (2011). Evaluation of efficacy of selected antibacterials in growth of methicillin resistant *Staphylococcus aureus* (MRSA) isolated from wounds and burns infection in Hilla city (An *in vitro* and *in vivo* study). MSc thesis. College of medicin, Babylon University.

[10] 1Anacarso, I., Condò, C., Sabia, C., Messi, P., de Niederhausern, S., Bondi, M. and IseppiR. (2013). Antimicrobial Resistance and Other Related Virulence Factors in *Staphylococcus* Spp isolated from Food, Environmental and Humans in Italy. Universal J. Microbiol. Res., 1(1): 1-9.

[11] Kearns, A. M., Seiders, P. R., Wheeler, J., Freeman, R. and Steward, M. (1999). Rapid detection of methicillin-resistant Staphylococci by multiplex PCR. J. Hospital Infect., 43(1): 33-37.

[12] Forbes, B. A., Daniel, F. S., and Alice, S. W. (2007). Bailey and Scott's diagnostic microbiology. (12th. edn.), Mosby Elsevier company, USA.

[13] Sarathbabu, R., Rajkumari, N. and Ramani, T. (2013). Characterization of Coagulase negative Staphylococci isolated from urine, pus, sputum and blood samples. Int J Pharm Sci Inven., 2: 37-46.

[14] Moura, T., Campos, F., Azevedo, P., Sand, S., Franco, A., Frazzon, J. and Frazzon, A. (2012). Prevalence of enterotoxin-encoding genes and antimicrobial resistance in coagulase-negative and coagulase-positive *Staphylococcus* isolates from black pudding. 45 :5.

[15] Mulu, W., Kibru, G., Beyene, G. and Damtie, M. (2012). Postoperative nosocomial infections and antimicrobial resistance pattern of bacteria isolates among patients admitted at Felege Hiwot Referral Hospital, Bahirdar, Ethiopia. Ethiopian J. Health Sci., 22(1): 7-18.

[16] Shinji, H., Yosizawa, Y., Tajima, A., Iwase, T., Sugimoto, S., Seki, K. and Mizunoe, Y. (2011). Role of fibronectin-binding proteins A and B in in vitro cellular infections and in vivo septic infections by *Staphylococcus aureus*. Infect Immun. 79(6):2215-23.

[17] Garzoni, C., Kelley, W. L. (2009).*Staphylococcus aureus*: new evidence for intracellular persistence. Trends Microbiol. 17(2): 59-65.

[18] Shinji, H., Yosizawa, Y., Tajima, A., Iwase, T., Sugimoto, S., Seki, K. and Mizunoe, Y. (2011). Role of fibronectin-binding proteins A and B in in vitro cellular infections and in vivo septic infections by *Staphylococcus aureus*. Infect Immun. 79(6): 2215-2223.

[19] Valle, J., Gomez, L., Piriz, S., Goyache, J., Orden, J. and Vadilo, S. (1990). Enterotoxins production by Staphylococcal isolated from healthy goats. Appl Environ Microbiol., 56(5): 1323-1326.

[20] Vasconcelos, N. G., Pereira, V. C., Araujo, J. P. and da Cuncha, L. R. (2011). Molecular detection of enterotoxins E, G, H and I in *Staphylococcus aureus* and coagulase-negative staphylococci isolated from clinical samples of newborns in Brazil. J Appl Microbiol., 111: 749-762.

[21] Daniel, B., Saleem, M., Naseer, G. and Fida, A. (2014). Significance of *Staphylococcus haemolyticus* in hospital acquired infections. J Pioneer Med Sci 4, 119-126.

[22] Raponi, G., Ghezzi, M. C., Gherardi, G., Dicuonzo, G., Caputo, D. and Venditti, M. (2005). Antimicrobial susceptibility, biochemical and genetic profiles of *Staphylococcus haemolyticus* strains isolated from the bloodstream of patients hospitalized in critical care units. J Chemother. 17(3):264-269.

[23] Mandal, A. M. D. (2016). *Staphylococcus aureus* Virulence Factors. J Life Sciences & Medicine. Vol.27-Issue 2: p50-56.

[24] Veras, J. F., Carmo, L. S., Tong, L. C., Shupp, J. W. and Jett, M. (2008). A study of the enterotoxigenicity of coagulase-negative and coagulase-positive staphylococcal isolates from food poisoning outbreaks in Minas Gerais, Brazil. Int J Infec Dis., 12(4): 410-415.

[25] Podkowik, M., Park, J. Y., Seo, K. S., Bystron, J. and Bania, J. (2013). Enterotoxigenic potential of coagulase-negative staphylococci. Intern J Food Microbiol. 163; 34-40.