Virulence Characterization of Coagulase-Negative Staphylococci Isolated from Sexually Active Women with Symptomatic Genital Tract Infection, In Comparison with *Staphylococcus aureus* Isolated from the Same Cases

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تحديد صفات الضراوة للمكورات العنقودية السالبة للكواكيوليز (Coagulase-Negative Staphylococci) المعزولة من نساء نشطات جنسيا مصابات بالتهاب المسلك التناسلي المصحوب بالأعراض، بالمقارنة مع المكورات العنقودية الذهبية (*S. aureus*) المعزولة من الحالات نفسها

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الخلاصة

تضمنت هذه الدراسة 33 عزلة للمكورات العنقودية السالبة للكواكيوليز (Coagulase-negative staphylococci: CNS) و 35 عزلة للمكورات العنقودية الذهبية (*Staphylococcus aureus*) عزلت من نساء نشطات جنسيا مصابات بالتهاب المسلك التناسلي المصحوب بالأعراض (64 حوامل و 110 غير حوامل). أظهرت مقارنة الصفات المظهرية عدم وجود فرق معنوي بين عزلات CNS و عزلات *S. aureus* بالنسبة لفعالية تحليل الدم (63.6% و 54.2%، على التوالي) و فعالية تحليل الدهن (87.8% و 88.5% ،على التوالي) وتكوين الغشاء الحيوي (96.9% و 94.2%،على التوالي) و فعالية تحليل البروتين (18.1% و 31.4%، على التوالي). بينما كان الفرق معنوي ($P \leq 0.05$) بالنسبة لفعالية تحليل اليوريا (18.1% و42.8% ،على التوالي). ما عدا فعالية تحليل الدهن وتكوين الغشاء الحيوي، اظهر التعبير المشترك لعوامل الضراوة وجود تعبير عكسي لفعالية تحليل الدم و فعالية تحليل البروتين لكل من عزلات CNS و*S. aureus* . بالنسبة لل CNS كان فقط 33/3 (9%) عزلات محللة للدم ومحللة للبروتين اما بالنسبة لعزلات *S. aureus* امتلكت هذه الصفة 35/2(5.7%) عزلة. كان هناك ترابط بين فعالية تحليل اليوريا وكل من فعالية تحليل الدم (20 %) و فعالية تحليل البروتين .(11.4%) ما بين عزلات *S. aureus* أكثر مما بين عزلات CNS (6.0% و3.0 % ،على التوالي). نستنتج بالنسبة للعوامل التي تناولتها هذه الدراسة ،إن ضراوة CNS المعزولة من نساء مصابات بالتهاب المسلك التناسلي، مقاربة لضراوة عزلات *S. aureus* التي تعد ممرض ضاري في تلك الإصابات. لذا فان CNS يمكن أن تعد ممرض انتهازي في هذه الحالات وليس مجرد ملوثات.

Abstract

This study included 33 coagulase-negative staphylococci (CNS) and 35 *Staphylococcus aureus* isolated from sexually active females with symptomatic genital tract infection (64 pregnant and 110 non-pregnant). Comparison of phenotypic characteristics showed that the difference is not significant between CNS and *S. aureus* isolates for hemolytic activity (63.6% and 54.2%, respectively); lipolytic activity (87.8% and 88.5%, respectively); protease production (18.1% and 31.4%, respectively). and biofilm formation (96.9% and 94.2%, respectively). Whereas the difference is significant ($P \leq 0.05$) for urease production (18.1% and 42.8%, respectively). With the exception of lipolytic activity and biofilm formation, co-expression of other factors showed that there was an opposite expression of hemolytic and proteolytic activities of both CNS and *S. aureus* isolates. For CNS only 3/33 (9%) of the isolates were hemolytic and preoteolytic while for *S. aureus* only 2/35 (5.7%) isolates had this characteristics. Also for *S. aureus* isolates, ureolytic activity was more correlated with hemolytic (20%) and proteolytic (11.4%) activities than in CNS (6.0% and 3.0%, respectively). We concluded that, for this study included factors, the virulence of CNS isolated from females' genital tract infection is comparable to that of the potential females' genital tract pathogen, *S. aureus*. So that, CNS can be opportunistic pathogens in these cases rather than mere contaminants.

Key words: coagulase-negative staphylococci, *S. aureus*, female genital tract infection, virulence factors.

Introduction

Staphylococci are a major human pathogens, causing a large variety of infections worldwide (1). *Staphylococcus aureus* is frequently isolated from community and hospital infections, including septicemia, lower respiratory, urinary tract, and skin infections (2, 3, 4), whereas coagulase-negative staphylococci (CNS) are most predominant cause of nosocomial bloodstream infections, cardiovascular infections, and infections of the eye, ear, nose, and throat. Tissue infections, the pathogenesis of which includes biofilm formation by CNS in absence of a foreign body, include endocarditis of native valves, and less frequently otitis media (3, 4, 5, 6, 7, 8). CNS also cause wound and urinary tract infections. (9). CNS are normal flora components of various parts of the skin and of the respiratory and gastrointestinal system mucosa of man; they may also appear in animals and foodstuff (3, 4, 8). The group of patients exposed to the risk of CNS-induced infections includes immune-compromised patients, and patients with inserted or implanted foreign materials (3, 4, 7, 8, 10). *S. aureus* is part of the normal human flora. The anterior nares is the most frequent site of human colonization, although the skin, vagina, axilla, perineum, and oropharynx may also be colonized (3, 10).

S. aureus produces numerous extracellular proteins and toxins. In addition, *S. aureus* has the capacity to adhere to catheters and other indwelling devices and

form a multicellular community, known as a biofilm (11, 12). CNS, as opposed to the *S. aureus* species are not equipped with such widespread spectrum of virulence factors. They may participate, as commensal flora, in the development of infections only when external barriers (e.g. skin) were damaged due to wounds, inoculation or implantation of foreign bodies (7, 8). In general, the factors that CNS need to survive in their habitat in or on the human body are likely the same that they need to efficiently colonize tissue during an infection. Success as pathogens in these cases depends on adhesion factors, evasion of the host's immune system, and the production of factors harmful to host tissue, such as toxins and degredative exoenzymes (7, 13). The ability of CNS to form a biofilm, mainly on the surface of foreign bodies in the human organism but also on the surface of tissues, plays the most important role in the pathogenesis of CNS-induced infections (8, 14). The study of CNS pathogenicity has also shown that various metabolites are produced by these microorganisms, including enzymes and toxins which may play a role in the pathogenicity of these microorganisms (15), such as lipases, proteases, other exoenzymes, which possibly contribute to the persistence of CNS in the host and may degrade host tissues and the production of hemolysin, , DNAse, and TNAse (7, 13).

There are presently 41 recognized taxons, designated Coagulase-negative staphylococci. Although long considered non-pathogens as the components of normal human skin and mucosa, lately they turned into significant etiological agents causing nosocomial infections, mainly in link with the presence of foreign bodies in the human organism (8). In addition, their increasing resistance to antibacterial drugs evoked deepening concern of infections involving CNS (3, 4, 8). Little is known about the virulence factors produced by CNS that contribute to the pathogenesis of infections caused by these microorganisms (13). The specific sites and frequency of infection seem to be related to the site and frequency of normal colonization (7). In our investigation of the bacterial causative agents of aerobic vaginitis in pregnant and non-pregnant women with symptomatic genital tract infection (unpublished data), we found that there is a high frequency of CNS isolation from these patients in a significant growth and most of them were isolated as single, pure culture. These findings raise a question about their significance as opportunistic pathogens of females' genital tract. So that we carried out this study for *in vitro* investigation of virulence factors of these isolates including: biofilm formation, and production of hemolysin, lipase, protease, and urease in comparison with *S. aureus* isolated from the same cases in which it is considered as potential and common pathogen.

Materials and Methods

Patients

This study included sexually active pregnant and nonpregnant women (aged 20 to 40 years) with symptomatic genital tract infection, attending private Obstetrics and Gynecology Clinic in Al-Kut/Wassit Province/Iraq and were, therefore, mainly symptomatic and self referred.

Specimen Collection and Processing

Specimens were collected during May 2008 to March 2010. High vaginal swabs were collected by the Gynecologist (16). and streaked immediately after collection on blood agar (Himedia) and eosine methylene blue agar (EMB) (Himedia) plates. The plates were incubated at 37 °C for 24-48 hours at ambient air.

Identification of the Isolates

Staphylococcal isolates initially identified by Gram staining, the catalase test, and susceptibility to 0.04 U bacitracin to characterize the genus *Staphylococcus* and the bacterial colonies were submitted to coagulase tests (17).

The isolates were identified biochemically to species level by using API Staph system (bioMerieux) according to the manufacturer's instructions.

Detection of hemolytic, lipolytic and proteolytic activity

These experiments were carried out according to Michelim *et al.* (18). For the evaluation of enzymatic activities, the isolates were initially grown on tryptic soy agar (TSA) at 37°C for 24 h. All the enzymatic experiments were repeated twice with three replications per experiment.

Hemolytic activity of the isolates was assessed after 48 h incubation at 37°C on TSA supplemented with 5% (v/v) human blood.

Lipolytic activity was assessed by streaking the isolates onto Tween 20 (T20) agar and incubating the plates at 37°C for 72 h. Lipase activity was determined by measuring the halos of precipitated Tween around the streaks.

Proteolytic activity was measured by inoculating the isolates onto modified TSA medium (18). After incubation the plates were flooded with a saturated solution of ammonium sulfate to precipitate the proteins in the medium, isolates being classified according to the size of the halo of protein hydrolysis.

Phenotype analysis of biofilm production on CRA

Biofilm forming colony morphology was detected for the isolates on Congo red agar (CRA) plates (19). Bacteria were cultured in 10 ml tryptic soy broth at 35°C for 24 h without shaking, then plated onto CRA plates. Incubation was carried out at 35°C for 24 h and an additional 24 h at room temperature before recording the colony morphology. Crusty black colonies with dry filamentous appearance were recorded as biofilm producers, smooth pink colonies as non-producers and intermediate colony morphology (pink with dark centers resembling bull’s eyes) as potential biofilm producers (20).

Statistical analysis

The results were analyzed statistically using Chi-square (21). A P value below 0.05 was considered to indicate statistical significance.

Results and Discussion

The choice of virulence factors

This study included 33 (19%) CNS and 35 (20.1%) *S. aureus* isolated from pregnant (64) and non-pregnant (110) women with symptomatic genital tract infection. Because of the small number of the isolates, we gathered both pregnant and non-pregnant isolates to get a clearer picture of the distribution of virulence factors of this study. Phenotypic virulence characterization of the isolates is shown in Table-1.

Table-1: Phenotypic virulence characterization of CNS in comparison with *S. aureus* isolated from females' genital tract infection.

Virulence factors	Number of positive isolates (%)	
	CNS (n) = 33	***S. aureus* (n) = 35**
Hemolytic activity	21 (63.6)	19 (54.2)
Urease production	6 (18.1)	15 (42.8)
Protease production	6 (18.1)	11 (31.4)
Lipase production	29 (87.8)	30 (85.7)
Biofilm formation	32 (96.9)	33 (94.2)

CNS: coagulase-negative staphylococci.

To our knowledge this is the first study in this respect. Previous studies have concentrated on studying the virulence factors of CNS (especially *S. epidermidis*) isolated from patients with nosocomial infections associated with foreign body use. In genital tract infections, CNS were considered as contaminants while *S. aureus* was known as a potential cause of genital tract infections, so that the possible pathogenic role (as opportunistic pathogens) of CNS in genital tract infections has been ignored. As they are part of normal vaginal flora, isolation of CNS from clinical cases is considered as contamination. Carson *et al.* (22) showed that most of the staphylococci isolates were CNS, which are perceived to be normal commensal organisms. The association of CNS species with illness may only reflect opportunity rather than virulence (23), while recently da Cunha *et al.* (13) reviewed that these microorganisms should not be ignored or classified as mere contaminants.

Biofilm formation, hemolytic activity, lipase, protease, and urease production were the principal pathogenicity factors of CNS as shown in previous studies (7, 24, 25, 26, 27). So that these factors we considered in this study as an

attempt to determine the pathogenic potential of these isolates in comparison with *S. aureus* (potential pathogen) isolated from the same cases.

Hemolytic activity of the isolates

Although the difference is not significant, hemolytic activity was more prevalent among CNS isolates (63.6%) than among *S. aureus* isolates (54.2%). Michelim *et al.* (18) found a significantly higher frequency of hemolytic activity in the clinical isolates of *S. epidermidis* collected at neonatal, pediatric and adult Intensive Care Units (ICUs) (37.7%) than in the community isolates (20%). da Cunha *et al.* (13) reported that the study of CNS isolated from infectious process of newborns revealed the production of toxins, including hemolysins (19.9%).

This is a good indication of virulence of these bacteria as hemolysis is strongly correlated with bacterial virulence especially in *S. aureus*. *S. aureus* is pathogenic to animals and humans and produce many virulence factors such as hemolysins which include alpha-, beta-, gamma- and delta-hemolysin. These agents play a very important role in staphylococcal pathogenesis. Hemolysins are cytolytic to a variety of host cells. Toxicity to immune cells makes them a good means for staphylococci to avoid phagocytosis and other forms of immune response(28). *S.aureus* α-toxin can have lethal effects, damages membranes (resulting in, among other things, hemolysis), and is responsible for a form of dermonecrosis (10). The role of hemolytic toxins in the pathogenesis of coagulase negative staphylococcal infections is far from clear because it is not known whether hemolysin production occurs in vivo and how the toxins might interact with other bacterial and host factors (29). Hemolysin production by different CNS species was reported by others (30, 31). Toxin production *in vitro* by CNS strains may reflect the severity of the infection (30). Hemolysis type β was higher in the vagina, this could be due to significant alterations in the microbiological environment of the vagina ecosystem (32), of women acquiring bacterial transmitted infections (33), including those staphylococci which have the ability to transfer from incompletely hemolytic into completely hemolytic (beta) according to change in the environment conditions (34).

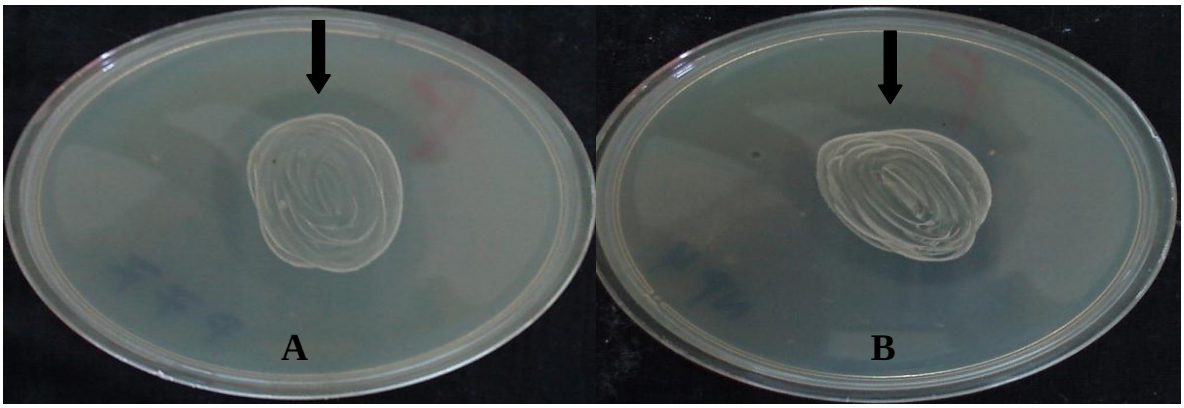
Urease production

Urease production is included in this study as it may be an important virulence factor that predisposes those patients with urease positive isolates to UTI. Urease production was significantly ($P \leq 0.05$) more prevalent among *S. aureus* isolates (42.8%) than among CNS isolates (18.1%). This means that *S. aureus* isolates are more probable to cause UTI than CNS. *S. aureus* is frequently isolated from community and hospital infections, including septicemia, lower respiratory, urinary tract, and skin infections (2, 3, 4). Several other extracellular components are secreted by *S. aureus* including coagulase, staphylokinase, lipase, urease, and hyaluronidase (4). Urease production by *S.simulans*, *S. capitis* subsp. *urealyticus*, *S. hominis*, *S. warneri*, and *S. caprae*, was also reported (8).

The urease which clearly functions as a virulence factor (35) causes alkalinization of the urine, which, in analogy to other lipases, may enhance the activity of the lipase (36). Urease is an important virulence factor that it contributes to invasiveness by damaging bladder tissues (7, 8).

Proteolytic activity of the isolates

Protease was produced by 18.1% of CNS isolates and by 31.4% of *S. aureus* isolates (Fig1). The difference is not significant. Michelim *et al.* (18) found that although the overall frequency of proteolytic isolates was 45.7% there was a significant difference between clinical isolates (49%) and community isolates (30%), indicating the importance of this factor in the *S. epidermidis* infectious process.



modified tryptic soy agar. Protein hydrolysis occurs as transparent halos around bacterial growth (arrows).

Although the difference is not significant between CNS and *S. aureus* isolates regarding protease production, this activity is more available among *S. aureus* isolates (31.4%) than among CNS isolates (18.1%). This is consistent with Otto (7) who reviewed that the degredative exoenzymes are less frequently found in CNS compared to *S. aureus*, which makes infections with CNS more silent than those with *S. aureus*. Kasper *et al.* (3) demonstrated that CNS are considerably less virulent than *S. aureus* but remain important pathogens in selected clinical settings. The role of protease in pathogenesis of *S. aureus* and of CNS is not well defined. It seems likely that protease may participate in distribution of infection by degrading proteins. Goguen *et al.* (37) demonstrated that proteases, like lipases, have been shown to be involved in tissue damage and the inflammatory response of the host as well as in the degradation of signal peptides altering the immune response (38). *S. aureus* proteases role in human disease pathogenesis is unknown(4). It is not known if the *S. epidermidis* proteases contribute to virulence by degrading host tissues, however this is to be expected. The serine protease is preferentially expressed in adherent culture, suggesting a possible role in biofilm formation. In another study, most likely the same protease has been reported to be able to degrade fibrinogen, complement protein C5, and several other proteins, suggesting a role in the escape from the immune defense system (7).

Lipolytic activity

Lipase was produced by 87.8% of CNS and by 85.7% of *S. aureus* isolates. The difference is not significant which indicates the importance of this enzyme, for both CNS and *S. aureus*, as a colonization factor of the skin and mucous membranes. Michelim *et al.* (18) reported that among the isolates of *S. epidermidis* the high majority (90%) were positive for lipase activity. There are no differences on the percentage of lipolytic isolates which were found when comparing clinical and community isolates, indicating that lipolytic activity is important not only during pathogenic processes but also for *S. epidermidis* skin colonization. Lipases from *S. saprophyticus*, *S. haemolyticus*, *S. hyicus*, *S. warneri*, and *S. xylosus* (7), and *S. simulans*, *S. capitis*, *S. hominis*, *S. schleferi*, *S. cohnii*, *S. warneri*, *S. caprae.* (8) have also been described. da Cunha *et al.* (13) showed that, except for *S. hominis* and *S.xylosus*, all spp. produced lipase.

The production of lipases is a common property of staphylococci (39). Gribbon *et al.* (40) showed that the contribution of these enzymes to virulence, however, is not clearly understood, although it has been suggested that lipases may be important for colonization and persistence of resident organisms on the skin. Lipases have been implicated as possible virulence determinants in the pathogenesis of a number of localized infections, such as boils or abscesses (41, 42), and studies utilizing *in vitro* expression technology have also indicated that lipases are produced during infections in a murine abscess model (43). Lipases may contribute to persistence of the microorganisms by providing a source of energy or by facilitating adherence (7, 40). It has also been suggested that lipase lowers the concentration of lipids that inhibit another staphylococcal enzymes (44). The recent finding that lipase of *S. epidermidis* can bind to collagen might constitute a novel role for lipase in virulence (7).

Biofilm formation

Most of this study isolates were biofim formers. There is no significant difference between CNS (96.9%) and *S. aureus* (94.2%) isolates. This very high percentage of isolates with this characteristic, indicates the critical importance of biofilm formation for colonization of genital tract mucous membranes especially if we know that most of the isolates were potential producers (Fig 2). By reviewing literatures, the pathogenic importance of this factor is not clear. Michelim *et al.* (18) showed that biofilm formation was found to occur with a significant difference between the clinical isolates of *S. epidermidis* collected at neonatal, pediatric and adult ICUs, (38.8%) and the community isolates (20%). Also Christensen *et al.* (45) reported that the frequency of adherent growth was significantly higher (63%) in strains associated with clinical signs of infection than in strains not associated with such signs (37%). The study of CNS isolated from infectious process of newborns revealed the production of slime in (17.1%) of all CNS samples isolated (13). In contrast to these researchers, Eftekhar and Mirmohamadi (46) found that 64% of patients (nosocomial infections, different

cases: blood, urine, surgical wounds, exudatees) and 68% of normal skin flora isolates had the potential to form biofilm, suggesting no difference between the two categories.

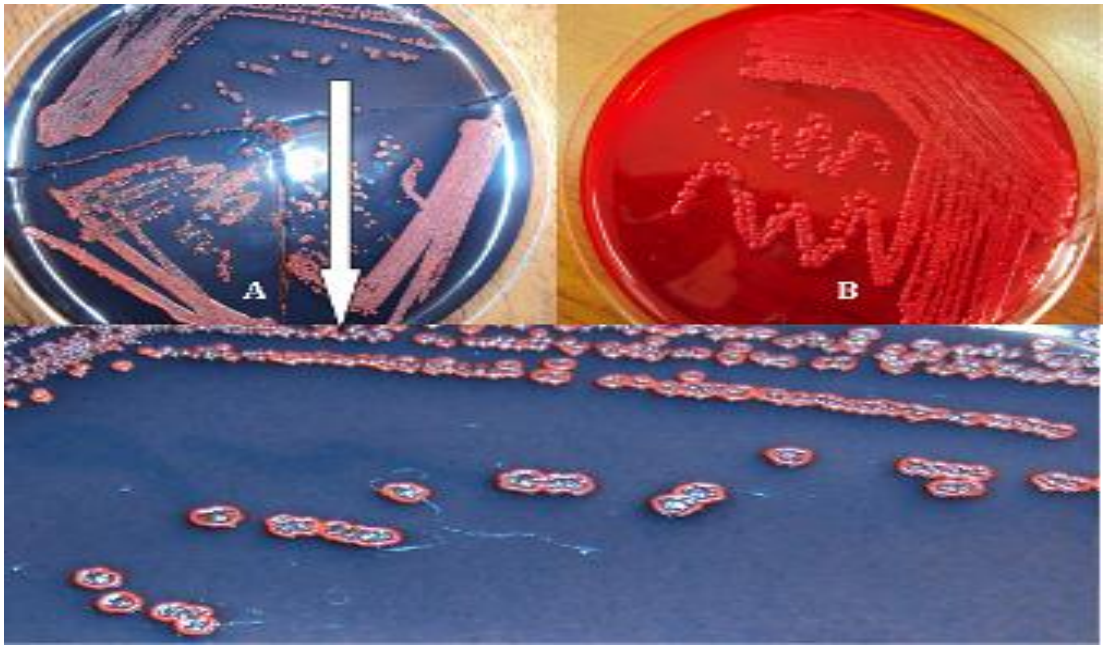


Fig. 2: Biofilm-positive CNS isolates (A) and biofilm-negative isolate (B) on CRA medium.

In the light of the results of this study, we agreed with the opinion that slime is a colonization and a virulence factor (45). The formation of a biofilm is an immense contribution to the successful colonization of host tissues and indwelling medical devices (3, 7, 8, 9, 10). Recently, Eftekhar and Mirmohamadi (46) concluded that *S. epidermidis* isolates from patients with symptomatic infections are not necessarily more virulent than the skin contaminants and the capacity to form biofilms *in vivo* is influenced by environmental stimuli independent of the *icaADBC* gene products. For other researchers there is a controversy. Some of them considered this property as a potential virulence factor (7, 8, 27, 47, 48) while others do not (14, 49, 50, 51).

Co-expression of virulence factors and the seriousness of these infections

With the exception of lipase and biofilm formation which were expressed by most isolates of both *S. aureus* and CNS, the co-expression of other factors is notable (Table-2). For both CNS and *S. aureus*, 100% of the isolates have one or more virulence factors. da Cuhna *et al*. (13) revealed the presence of one or more virulence factors in 77.8% of the CNS strains, suggesting that CNS virulence factors provide a selective advantage for skin colonization of hospitalized newborns.

Table-2: Co-expression of virulence factors by CNS and *S. aureus* isolated from females' genital tract infection.

Combined virulence factors	No. of positive isolates (%)	
	CNS (n) = 33	***S. aureus* (n) = 35**
All five factors	2 (6.0)	2 (5.7)
Four factors	4 (12.1)	7 (20.0)
Three factors	19 (57.5)	18 (51.4)
Two factors	4 (12.1)	8 (22.8)
One factor	4 (12.1)	None
Null	None	None

CNS: coagulase-negative staphylococci.

With the exception of lipolytic activity and biofilm formation, what is notable here is the opposite expression of hemolytic and proteolytic activities of both CNS and *S. aureus* isolates. For CNS only 3/33 (9%) of the isolates were hemolytic and proteolytic while for *S. aureus* only 2/35 (5.7%) isolates had this characteristic (Table-3). Both factors, as shown by other researchers who were mentioned here above, participate in the spread of infection by damaging host tissues, so that both *S. aureus* and CNS isolates are invasive and had comparable virulence as 54.2% and 31.4% of *S. aureus* isolates were hemolytic and proteolytic, respectively. As well as, 63.6% and 18.1% of CNS isolates were hemolytic and proteolytic, respectively. This means that about 80% of *S. aureus* and CNS isolates had the capacity to degrade host tissues and spread throughout the host body. Also, biofilm formation make these bacterial infections of long duration and difficult to be removed. Most of this study patients, as explained by the Gynecologists, had recurrent infection and although did have treatment, there were no symptom relief. As well as, most of this study isolates were resistant to most commonly used antibiotics (unpublished data). These results indicate the seriousness of these infections and the requirement for more detailed clinical and laboratory-based investigation both microscopically and culturally to determine the degree of these infections' risk and to describe the appropriate treatment. Also for *S. aureus* isolates, ureolytic activity is more correlated with hemolytic (20%) and preoteolytic (11.4%) activities than in CNS (6.0% and 3.0%, respectively). This may give us an indication about the potential of uropathogenic isolates and the importance of hemolytic and ureolytic activities in these infections. Confirmation of this assumption required a comparison study of these virulence factors for both CNS and *S. aureus* isolated from UTI and those isolated from genital tract infections.

Table-3: Co-expression of hemolytic, preteolytic and ureolytic factors by CNS and *S. aureus* isolated from females' genital tract infection.

Virulence factors	No. of positive isolates (%)	
	CNS (n) = 33	***S. aureus* (n) = 35**
Only hemolytic	16 (48.4)	10 (28.5)
Only proteolytic	2 (6.0)	5 (14.2)
Only ureolytic	1 (3.0)	2 (5.7)
Hemolytic and proteolytic	3 (9.0)	2 (5.7)
Hemolytic and ureolytic	2 (6.0)	7 (20)
Ureolytic and Proteolytic	1 (3.0)	4 (11.4)
Hemolytic, proteolytic and ureolytic	2 (6.0)	2 (5.7)

CNS: coagulase-negative staphylococci.

This co-expression of virulence factors indicate the important role of these factors together in pathogenesis of females' genital tract infection. After colonization, staphylococci replicate at the initial site of infection, elaborating enzymes that include serine protease, hyaluronidase, thermonuclease, and lipase. These enzymes facilitate bacterial survival and local spread across tissue surfaces, although their specific role in infections is still not well defined (3). We do not know much about the virulence factors of most CNS other than *S.epidermidis*, although some of them can cause similarly sever, or even more serious infections (as for ex. *S. lugdunensis*) (7). In addition to mechanisms surrounding device-associated infection, CNS are also known to express an increasing number of other virulence factors to varying degrees among the different species. These include hemolysins, phosphotases, thermonucleases, lipases, galactosidases, pyrrolidonyl arylamidases and various decarboxylases (7, 8).

We concluded that, for this study included factors, the virulence of CNS isolated from females' genital tract infections is comparable to that of the potential females' genital tract pathogen, *S. aureus*. *In vivo* pathogenesis study and toxin profile determination, are required to confirm these results.

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Recived ……..……. (1/8 /2010)
Accepted ……...……... (4/10/2010)