Evaluation of Virulence of Coagulase-Negative Staphylococci, Isolated from Sexually Active Women with Symptomatic Genital Tract Infection *in Vitro*

تقييم ضراوة المكورات العنقودية السالبة للكواكيوليز (Coagulase-Negative Staphylococci) المعزولة من نساء نشطات جنسيا مصابات بالتهاب المسلك التناسلي المصحوب بالأعراض مختبريا

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الخلاصة

تضمنت هذه الدراسة 33 عزلة للمكورات العنقودية السالبة للكواكيوليز (Coagulase-negative staphylococci: CNS) ، عزلت من نساء نشطات جنسيا مصابات بالتهاب المسلك التناسلي المصحوب بالأعراض (64 حوامل و 110 غير حوامل). أظهرت دراسة الصفات المظهرية أن 63.6% من عزلات CNS محللة للدم وان 87.8% محللة للدهن و96.9% مكونة للغشاء و18.1% منتجة لانزيم اليوريز و18.1% منتجة لانزيم البروتييز. باستثناء فعالية تحليل الدهن وتكوين الغشاء الحيوي، اظهر التعبير المشترك لعوامل الضراوة وجود تعبير عكسي لفعالية تحليل الدم و فعالية تحليل البروتين، إذ كان فقط 33/3 (9%) عزلة محللة للدم و محللة للبروتين. نستنتج بالنسبة للعوامل التي تناولتها هذه الدراسة ، إن ضراوة CNS المعزولة من نساء مصابات بالتهاب المسلك التناسلي، جديرة بالملاحظة ويمكن أن تعد CNS ممرضات انتهازية في هذه الحالات وليس مجرد ملوثات.

Abstract

This study included 33 coagulase-negative staphylococci (CNS) isolated from sexually active females with symptomatic genital tract infection (64 pregnant and 110 non-pregnant). Phenotypic virulence characterization showed that 63.6% of CNS isolates were hemolytic; 87.8% were lipolytic; 96.9% were biofilm formers; 18.1% were urease producers; and 18.1% were proteolytic. With the exception of lipolytic activity and biofilm formation, co-expression of other factors showed that there is an opposite expression of hemolytic and proteolytic activities. Only 3/33 (9%) of the isolates were hemolytic and proteolytic. We concluded that, for this study included factors, the virulence of CNS isolated from females' genital tract infection, is notable and that CNS can be opportunistic pathogens in these cases rather than mere contaminants.

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

Introduction

Staphylococci are major human pathogens, causing a large variety of infections worldwide (1). 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 (2, 3, 4, 5, 6, 7). CNS also cause wound and urinary tract infections. (8). 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 (2, 3, 7). The group of patients exposed to the risk of CNS-induced infections includes immune-compromised patients, and patients with inserted or implanted foreign materials (2, 3, 6, 7, 9).

CNS, as opposed to the *S. aureus* species are not equipped with 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 (6, 7). 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 (6, 10). 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 (7, 11). The study of CNS pathogenicity has also shown that various metabolites are produced by these microorganisms, including enzymes such as lipases, proteases, DNAse, and TNAse, and the production of hemolysin and toxins (6, 10, 12).

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 (7). In addition, their increasing resistance to antibacterial drugs evoked deepening concern of infections involving CNS (2, 3, 7). Little is known about the virulence factors produced by CNS that contribute to the pathogenesis of infections caused by these microorganisms (10). The specific sites and frequency of infection seem to be related to the site and frequency of normal colonization (6). 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 arise 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.

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 (13). 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 (14).

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.* (15). For the evaluation of

enzymatic activities, the isolates were initially grown in 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. Isolates being classified according to the diameter of the halos of hemolysis.

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. 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 (16). Bacteria were cultured in 10 ml tryptic soy broth at 35°C for 24 h without shaking, and were 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 (17).

Results and Discussion

The choice of virulence factors

This study included 33 CNS (19%) 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 this study virulence factors. Phenotypic virulence characterization of the isolates is shown in Table-1.

Table-1: Phenotypic virulence characterization of CNS isolated from females' genital tract infection.

Virulence factors	Number of CNS positive isolates (%) (n) = 33
Hemolytic activity	21 (63.6)
Urease production	6 (18.1)
Protease production	6 (18.1)
Lipase production	29 (87.8)
Biofilm formation	32 (96.9)

CNS: coagulase-negative staphylococci.

The principal pathogenicity factors of CNS were biofilms (18, 19), hemolysins (19, 20), lipases and proteases (19, 21). Urease was considered as virulence factor in urinary tract infections (UTI) (6). So that these factors we considered in this study as an attempt to determine the pathogenic potential of these isolates. 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, 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 (10) reviewed that these microorganisms should not be ignored or classified as mere contaminants.

Hemolytic activity of the isolates

Hemolytic activity was observed in 63.6% of CNS isolates. Michelim *et al.* (15) found a significantly higher frequency of hemolytic activity in the clinical isolates of *Staphylococcus epidermidis* collected at neonatal, pediatric and adult ICUs (37.7%) than in the community isolates (20%). da Cunha *et al.* (10) 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. For example, in *S. aureus* these agents play a very important role in 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 (24). 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 (25). Toxin production *in vitro* by CNS strains may reflect the severity of the infection, as one strain of *S. epidermidis* which elaborated the highest titers of hemolysins also had the lowest LD_{50} (26). All *S. haemolyticus* strains produced hemolysins in vitro, suggesting that hemolysin may contribute to the high virulence of this species (26). *Staphylococcus cohnii* ssp. *cohnii* and *S. cohnii* ssp. *urealyticus* are a coagulase-negative staphylococci considered for a long time as unable to cause infections. This situation changed recently and pathogenic strains of these bacteria were isolated from hospital environments, patients and medical staff. Several synergistic peptide hemolysins produced by these bacteria and acting as virulence factors responsible for hemolytic and cytotoxic activities (27). Hemolysis type β was higher in the vagina, this could be due to significant alterations in the microbiological environment of the vagina ecosystem (28), of women acquiring bacterial transmitted infections (29), including those staphylococci which have the ability to transfer from incompletely hemolytic into completely hemolytic (beta) according to change in the environment conditions (30).

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 was produced by 18.1% of CNS isolates. Urease production by *S.simulans, S. capitis* subsp. *urealyticus, S. hominis, S. warneri*, and *S. caprae*, was reported (7). The first widely accepted pathogenic role of CNS was the association of *S. saprophyticus* with UTIs in women (3). CNS also cause wound and urinary tract infections. (8). The urease which clearly functions as a virulence factor (31) causes alkalinization of the urine, which, in analogy to other lipases, may enhance the activity of the lipase (32). Urease is an important virulence factor for *S. saprophyticus* in that it contributes to invasiveness by damaging bladder tissues (6, 7).

Proteolytic activity of the isolates

Protease was produced by 18.1% of CNS isolates (Fig. 1). Michelim *et al.* (15) 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.

The role of protease in pathogenesis of CNS is not well defined. It seems likely that protease may participate in distribution of infection by degrading proteins. Goguen *et al.* (33) 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 (e.g. human neutrophil proteins (HNPs), antimicrobial platelet proteins, antibodies) altering the immune response (34). It is not known if the *S. epidermidis* proteases contribute to virulence by degrading host tissues, however this is to be expected. Three proteases have been described in *S. epidermidis* with different substrate

specificities and mechanisms of action. 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 (6).

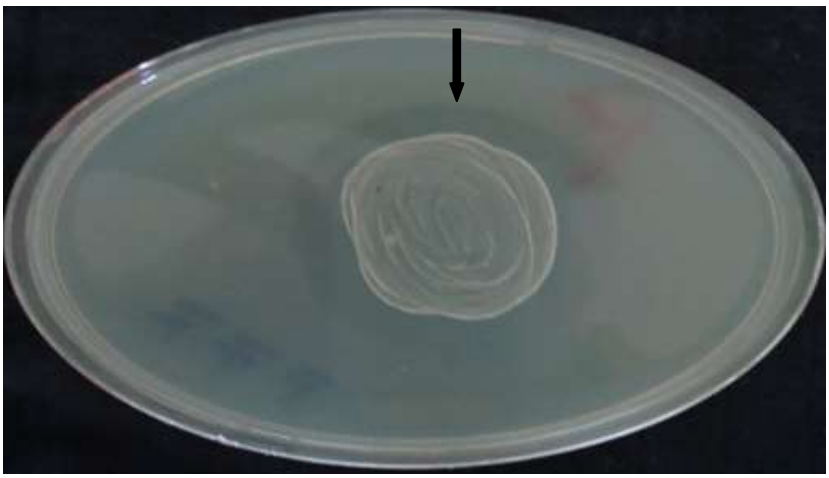

Fig. 1: Protease production by a CNS isolate on modified tryptic soy agar. Protein hydrolysis occurs as transparent halo around bacterial growth (arrow).

Lipolytic activity

Lipase was produced by 87.8% of CNS isolates. This high rate of lipase production indicates the importance of this enzyme as a colonization factor of the skin and mucous membranes. Michelim *et al.* (15) reported that among the isolates of *S. epidermidis* the high majority (90%) were positive for lipase activity. There is no differences on the percentage of lipolytic isolates 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. Extracellular lipases have been shown to be a pathogenicity factor of various microorganisms, including *S. epidermidis* (21), contributing for the survival of microorganisms in environments containing high concentrations of lipids, and affecting the capacity of the microorganism to penetrate the skin and invade epidermal tissues. Lipases from *S. saprophyticus*, *S. haemolyticus, S. hyicus, S. warneri,* and *S. xylosus* (6), and *S. simulans*, *S. capitis*, *S. hominis*, *S. schleferi*, *S. cohnii*, *S. warneri, S. caprae.* (7) have also been described. da Cunha *et al.* (10) showed that,except for *S. hominis* and *S.xylosus*, all spp. produced lipase.

The production of lipases is a common property of staphylococci (35). Gribbon *et al.* (36) 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, possibly in relation to nutrition or by the release of free fatty acids which may promote adherence. Lipases have been implicated as possible virulence determinants in the pathogenesis of a number of localized infections, such as boils or abscesses (37, 38), and studies utilizing *in vitro* expression technology have also indicated that lipases are produced during infections in a murine abscess model (39). Lipases may contribute to persistence of the microorganisms by providing a source of energy or by facilitating adherence (36). It has also been suggested that lipase lowers the concentration of lipids that inhibit another staphylococcal enzymes, the fatty acid-modifying enzyme (40). Lipases might contribute to virulence by enabling the bacteria to persist in the fatty secretions of the human and mammalian skin, and possibly interfering with phagocytosis. The recent finding that lipase of *S. epidermidis* can bind to collagen might constitute a novel role for lipase in virulence (6).

Biofilm formation

Most of this study isolates were biofim formers (96.9%). 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.* (15) 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.* (41) 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 (10). In contrast to these researchers, Eftekhar and Mirmohamadi (42) 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.

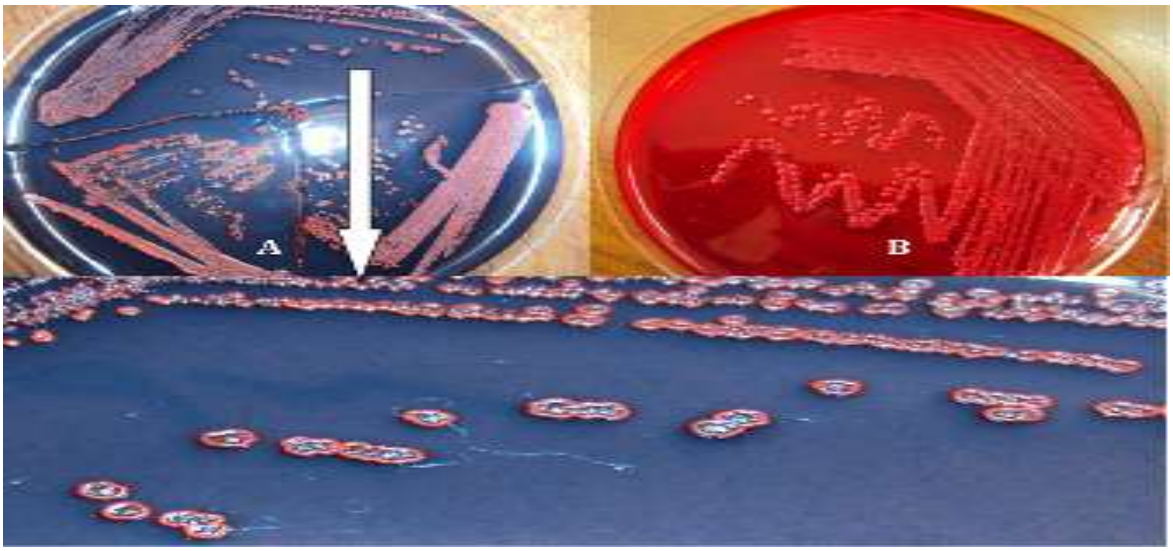

Fig. 2: Biofilm-positive CNS isolates (A) and biofilm-negative isolate (B) on CRA medium.

In the light of this study results, we agreed with the opinion that slime is a colonization and a virulence factor (41). The formation of a biofilm is an immense contribution to the successful colonization of host tissues and indwelling medical devices (2, 6, 7, 8, 9). Recently, Eftekhar and Mirmohamadi (42) 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 controversial, some of them considered this property as a potential virulence factor while others do not. Gotz, (43); and Mairo-Litran *et al*. (44) considered that the formation of biofilm represents an important virulence factor of certain strains of *S. epidermidis* and *S. aureus*. Biofilm formation has a very strong influence on the host's capacity to clear a CNS infection. The "slime" substance is thought to play a similar role as the *S. aureus* capsular polysaccharide, i.e. to decrease the phagocytic activity. In fact slime reduces phagocytic activity of murine macrophages (6). 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 and was associated with symptomatic infections (7, 23).

On the other hand, Vogel *et al*. (11) and Rodney and Costerton (45) suggested that slime production does not seem to be an important virulence factor of *S. saprophyticus* isolated from women with UTI. Baddour *et al.* (46) and Patrick *et al.* (47), in various experiments using animal models, the authors found no association between slime production and the development of endocarditis, suggesting that this polysaccharide is not a critical determinant of virulence and that other factors other than slime-mediated colonization determine the pathogenicity of these microorganisms.

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 this study, the co-expression of other factors is notable (Table-2).

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

Combined virulence factors	No. of positive isolates (%) (n) = 33
All five factors	2 (6.0)
Four factors	4 (12.1)
Three factors	19 (57.5)
Two factors	4 (12.1)
One factor	4 (12.1)
Null	None

CNS: coagulase-negative staphylococci.

Hundred percent of this study 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. With the exception of lipolytic activity and biofilm formation, what is notable here is the opposite expression of hemolytic and proteolytic activities. Only 3/33 (9%) of the isolates were hemolytic and proteolytic. (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 this study' CNS isolates are invasive, as 63.6% and 18.1% of these isolates were hemolytic and proteolytic, respectively. This means that about 80% of these 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. Also 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.

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

Virulence factors	No. of positive isolates (%) (n) = 33
Only hemolytic	16 (48.4)
Only proteolytic	2 (6.0)
Only ureolytic	1 (3.0)
Hemolytic and proteolytic	1 (3.0)
Hemolytic and ureolytic	2 (6.0)
Ureolytic and Proteolytic	1 (3.0)
Hemolytic, proteolytic and ureolytic	2 (6.0)

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 (2). 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*) (6). 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 (6, 7).

We concluded that, for this study included factors, the virulence of CNS isolated from females' genital tract infection is notable. So that, CNS can be opportunistic pathogens in these cases rather than mere contaminants. Further *in vivo* pathogenesis study and toxin profile determination, are required to confirm these results.

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