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STUDY OF SOME VIRULENCE FACTOR OF CO-AGULASE NEGATIVE *STAPHYLOCCUS* (CONS) ISOLATED FROM URINARY TRACT INFECTIONS (UTI)

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ABSTRACT

coagulase negative staphylococci (CONS) are now well established as major nosocomial pathogens associated with infections of indwelling medical devices, a total of 645 clinical samples, taken from different sources include blood, catheter urine specimen, wound and burn swabs and swabs of skin and nasal hospital staff, these sample had been cultured then isolated and identified pure isolates of different genus of bacteria, results show 85 (19.9%) were *Pseudomonas spp* isolates, 73 (17%) belong to Coagulase Negative Staphylococci (CONS) (*S. epidermidis*, *S. saprophyticus*, *S.Lentus*, *S. haemolyticus* and *S.hominis* constituted 68.5%, 13.7%, 6.85%, 6.85% and 4.1% Respectively), followed by both *S. aureus* and *E. coli* (*n* = 70,16.4%), followed by *Klebsiella* sp. (*n* = 68, 16%), *Proteus* sp. (*n* = 55,12.9%), Serratia marcescens (*n* = 6, 1.4%) isolated from study clinical samples. Second most important microorganism which isolated were Coagulase negative Staphylococci (*n* = 73, 17%), *Staphylococcus epidermidis* was mainly isolated from study patients group (68.5%) All isolates of CONS species are positive gram stain, produce white pigment except *S.saprophyticus* which produced yellow pigment and all isolates gave negative result of oxidase test, coagulase and catalase test was performed for all the isolates and all of them produced catalase enzyme that differentiates *Staphylococcus* from the genus *Streptococcus*, so *Staphylococcus* spp. reduce nitrate to nitrite and all isolates were identified by using epi staph and VITEK-2 system within 6 h., CONS are novobiocin resistance except

S.saprophyticus. staph.epidermidis isolated were none fermented xylose, sorbitol, melibiose while fermented many sugar as positive results for glucose, fructose, sucrose, maltose, lactose and mannose, in addition to produce urease, lipase and alkaline phosphatase while it cannot produce DNase and lecithinase.

MATERIAL AND METHODS

All Swabs from clinical source (blood, catheter urine specimen, wound and burn swabs as well as swabs of skin and nasal hospital staff were cultured by streaking on blood and MacConkey agar, plates were incubated aerobically overnight at 37ºC, if no growth was detected, plates were re-incubated for another 24 hours before reported negative cultures.For blood samples, 2 ml each was injected into blood culture bottles and incubated at 37°C in an automated blood culture system (Bactec 9120 system). They were identified as follows macroscopic observations (Culture Characteristics) as colonial morphology of grown bacteria on culture media, Colony size, color, elevation, edges, hemolysis on blood agar (Fischbach, 2001) and according to Fischbach (2001) were staining the isolated bacteria and culture Growth on mannitol salt agar to isolation of staphylococci, so this medium is used for motility and mannitol fermentation test. Catalase test: Pure isolated colonies (suspected S.epidermidis) picked up by sterile wooden stick, on microscope slide, using a dropper, place 1 drop of 3% H_2O_2 onto the organism on the microscope slide and observe for immediate bubble formation (O_2 + water = bubbles), indicating a positive test, negative result is no bubbles or only a few scattered bubbles. Beark down of H2O2 into O2 and H2O is immediate by catalase enzyme that produced by staphylococci, this test performed to differentiate between staphylococci (positive) and streptococci (negative) [Harley and Prescott, 2007].

Coagulase test (slide method): This method was used for rapid diagnosis, as the following: divide the slide into two sections with grease pencil. One should be labeled as “test” and the other as “control, one drop of distilled water was placed on each area of divided slide, then emulsify one or two colonies of Staphylococcus isolated on blood agar plate on each drop to make a smooth suspension and the test suspension is treated with a drop of citrated plasma and mixed well, Clumped within 5-30 seconds is taken as positive results[Vandepitte, 2003].

IMVIC Test: Indole test: this test was performed by inoculating the microorganism into peptone water, incubated for 24-48 hours at 37ºC; 0.5 ml of Kovac s reagent was then added. The broth was shaken well and examined after one minute. The formation of a red color ring in the reagent layer indicates indole positive, and yellow ring indicates a negative result.

Methyl red test: this test is employed to detect the production of sufficient acid during the fermentation of glucose, after incubation period at 37ºC, 5 drops of the reagent were added, the red color indicates a positive test.

Voges-proskauer (VP) test: This test is used to detect acid accumulation during glucose fermentation; a positive reaction was indicated by presence of a pink color during 15 minutes.

Citrate utilization test: a positive citrate utilization test indicated by the change of indicator color (bromothymol blue) from green to blue color after inoculation of the Simmon´s citrate agar with the tested microorganism.

Kligler iron agar: a positive fermentation of dextrose and lactose was indicated by the change of phenol-red indicator color from red to yellow and raised the medium as a result of gas production and some microorganisms produce hydrogen sulphide (H_2S) from thiosulphate. The H_2S reacts with iron-salts to produce a black precipitate.

Urease activity: urease production was tested by inoculating the tested bacteria onto the tube containing urea agar slant and incubated at 37ºC for 18-24 hours, a pink color is an indicator for positive urease activity.

DNase production: DNase agar was heavily streaked with the bacteria then incubated for 18- 24hrs at 37°C. Flood the plate with Hydrochloric Acid. Leave the plate to stand for a few minutes to allow the reagent to absorb into the plate. Decant excess hydrochloric acid and then examine the plate within 5 minutes.

Novobiocin sensitivity test (5µg/disc): this test was used to distinguish among Staphylococcus spp. An overnight bacterial culture broth was spread over a plate of Mueller-Hinton agar (2-1-5) by sterile swab. A disc of Novobiocin 5µg was placed on the agar medium. Then the plates were incubated at 37ºC for 24 hours and the inhibition zone around the disc was measured (large zone of inhibition over 15 mm in diameter or smaller inhibition zone) [Collee et al., 1996].

Sugars fermentation: determine the utilization of the sugars glucose, fructose, sucrose, maltose, lactose, xylose, mannose, sorbitol, melobiose, nitrate reduction, presence of urease, For the sugar fermentation test, commercially available disks specific for each sugar were

placed in tubes containing 2.5 ml Purple Broth Base medium, readings after 24, 48 and 72 h of incubation at 37^{O}C. [Kloos and Schleifer, 1994].

API staph System: Is an identification system for staphylococci, The API staph strip consists of 20 micro tubes containing dehydrated substrates, these tubes were inoculated with bacterial suspension which reconstitutes the media, during incubation, metabolism produces color change either spontaneously or revealed by the addition of reagents (Fischbach, 2001).

Vitek 2 system: all S. epidermidis isolates were characterized using vitek 2 compact. The VITEK 2 is an automated microbial identification system. With its colorimetric reagent cards, and associated hardware and software advances, the VITEK 2 offers a state-of the art technology platform for phenotypic identification methods.

RESULTS AND DISCUSSION

Many of pathogens are part of endogenous flora but some may have been acquired by contamination from hospital staff or by contaminated solutions or non-sterile equipment or from other patients.

Table 1: Number and percentage of bacteria isolated from different patients.

Bacterial isolated	Number and percent of bacteria isolates (from 645 patients)	
	Number	***%***
Pseudomonas spp	85	19.9
Coagulase negative Staphylococci	73	17
Staphylococcus.aureus	70	16.4
Escherichia .coli	70	16.4
Klebsiella sp.	68	16
Proteus. Sp.	55	12.9
Serratia marcescens	6	1.4
culture isolated	427	
Non-culture isolated	218	
Total	645	

Results in table. 1 showed samples cultures revealed 427 had bacterial isolates from 645 pateints (while 218 non- culture isolate), the most predominant pathogen was *Pseudomonas spp* (*n* = 85, 19.9%), the second most important microorganism which was isolated Coagulase negative Staphylococci (*n* = 73, 17%) followed by both *S. aureus* and *E. coli* (*n* = 70, 16.4%), followed by *Klebsiella* sp. (*n* = 68, 16%), *Proteus* sp. (*n* = 55, 12.9%) and *Serratia marcescens* (*n* = 6, 1.4%) isolated from study groups (blood samples, urine catheters

and wound and burn swab as well as swab of skin and nasal of hospital staff).

Pseudomonas spp were the most common pathogen isolated from this study groups (*n* = 85, 19.9%), which had became as important causing of infections, especially in patients with compromised host defense mechanism, these study results were fully in line with the results of Nwankwo (2014) who founded bacteria appeared *Pseudomonas spp*. was commonest pathogen isolated from urine of patients with indwelling urethral catheter.

These results also agreed with Singh *etal.*, (2003) whom found *Pseudomonas* species was most commonly isolated organisms from burn patients were followed by *S. aureus* and *Klebsiella* species so these results were also in agree with other studies [Ozumba and Jiburum 2000], while Mama *etal.*, (2014) showed the predominant bacteria isolated from infected wounds were *Staphylococcus aureus* 47 (32.4%) followed by *Escherichia coli* 29 (20%), Proteus species 23 (16%), Coagulase negative Staphylococci 21 (14.5%), *Klebsiella pneumoniae* 14 (10%) and *Pseudomonas aeruginosa* 11 (8%). These bacteria may enter the bladder through contamination of the tip during insertion with the flora of the distal urethra or from bacteria ascending outside or the inside of catheter, also Getliffe (2008) showed in his study residual urine in the bladder of catheterized patients increases the risk of bacteriuria, so wound infection is one of the health problems that are caused and aggravated by invasion of pathogenic organisms [Mama *etal.*, 2014].

Bacteria, which ranked second in this study, were the Coagulase negative staphylococcus 73(17%) from 427 isolated as follows Staphylococcus epidermidis was mainly isolated from Coagulase negative *staphylococcus* represented 68.5%.

Table 2: Biochemical tests and Novobiocin test for Coagulase negative Staphylococci and *Staph.aureus*

Coagulase negative and S.aureus	Biochemical tests					
	Pigment produce	Catalase	Coagulase	Oxidase	Nitrate reducing	Novobiocin resistance
Staph.epidermidis	White	+	-	-	+	-
Staph.saprophyticus	Yellow	+	-	-	+	+
Staph.lentus	White	+	-	-	+	-
Staph.heamolyticus	White	+	-	-	+	-
Staph.hominis	White	+	-	-	+	-
Staph. aureus	White	+	+	-	+	-

In table 2 the results showed all isolates of CONS species were produced white pigment excepted *S. saprophyticus* which produce yellow pigment and all isolated gave negative result of the oxidase test, coagulase and novobiocin resistance excepted *S. saprophyticus*, the catalase test was performed for all the isolates and all of them produced catalase enzyme that differentiates *Staphylococcus* from the Genus *Streptococcus* which gives negative result of the catalase test, additionally nitrate reduction test was performed for further identification because *Staphylococcus spp*. often reduce nitrate to nitrite. The biochemical features of CONS species were corresponding with the identification table of *API Staph* system, while Piccolomini *etal.,* (1994) Showed in his study that low agreement between the API Staph and biochemical test for identification of CONS which can be explained by the use of different incubation times, substrate concentrations and/or sensitivity markers.

Couto *etal.*,(2001) and Thorberg *etal.*,(2009) showed that the conventional biochemical analysis for the determination of *staphylococcus* at the species level appears to be expensive, laborious and time consuming although it is considered as gold standard for the identification of *staphylococcus* species. So all Isolates were identified by using API Staph and VITEK-2 system according to the manufacturer's instructions (see in material and methods), within 6 h., Vitek 2 correctly identified the commonly isolated strains; however, the limitations of the method may lead to ambiguous findings [Thiago *etal.*, 2014]. The ability of the VITEK 2 system to accurately give a rapid identification of clinically significant bacteria by Garrote *etal.*, (2000), the results of Layer *etal.*, (2006) showed API STAPH revealed more correct for identification of CNS, compared to VITEK systems, moreover the results indicated by Matthews *etal.*,(1990) showed that agreements of Vitek and API systems with conventional methods were 44.6% and 80.8% respectively, although additional tests were also required for final identification.

Table 3: Coagulase negative Staphylococci groups isolated from different clinical specimens.

BACTERIAL ISOLATED	NUMBER	%
S.epidermidis	50	68.5
S.saprophyticus	10	13.7
S.lentus	5	6.85
S.heamolyticus	5	6.85
S.hominis	3	4.1
Total	73	100

Results in table. 3 indicated that *S. epidermidis* was mainly isolated from study patients group (68.5%), Staphylococcus saprophyticus 13.7%, both *S.Lentus* and *S. haemolyticus* were 6.85% while *S.hominis* was 4.1%.

S.epidermidis is most commonly CONS isolated but other CONS species have been shown to cause nosocomial, This variation in the frequency rates may be due to variations in both environmental conditions and attitudes toward management also geographical variations, or the types and severity of infection included in the studies.

Coagulase negative staphylococci (CONS) have been identified as the etiological agent in various infections so among the microorganisms most frequently isolated in nosocomial infections also it cause wound and urinary tract infections [Cunha *et al*., 2006], whilst Al-Muhanna *etal.,* (2014) reported that (53%) of *S. haemolyticus*, (26%) *S. epidermidis* and (21%) *S.hominis* were the most commonly isolated CONS species from different clinical samples obtained from Iraqi patients undergoing catheter related infections, while Al-Dahmoshi *etal.,* (2013) demonstrated that common type of bacterial CONS species from Patients in Hilla City were both *Staphylococcus epidermides* and *Staphylococcus saprophyticus* (35.7%), While Begum *etal.* (2007), study showed *S. haemolyticus* (28.33%), *S.epidermidis* (26.67%) and *S. saprophyticus* (18.33%) were the most commonly isolated CONS species and this result is not significantly different from other reports where *S. epidermidis* was the highest isolated followed by *S. haemolyticus* in clinical infections, but Ali *etal.,* (2009) showed in his study 60% Coagulase negative *Staphylococci,* which showed 27 isolates (45%) regards *S. epidermidis*, 13 isolates (21.66%) *S. saprophyticus,* 10 isolates (16.67%) *S. xylosus*, 3 isolates (5%) *S. lentus,* two isolates (3.33%) *S. heamolyticus* and one isolate (1.67%) *S. simulans* and other one isolate of *S. hominis*, this is similar to a work done by Adeleye *etal.,* (2010); Akinkunmi and Lamikanra,(2010) who showed isolated similar CONS species from clinical samples.

S. epidermidis was the most isolated specie of CONS implicated in wound infections and this is in accordance with similar works earlier reported [Duran *et al*., 2010], whilst study of Azuka and Idahosa, (2013) Showen most commonly isolated species were S.*haemolyticus (*28.3%), *S.epidermidis (*26.7%) and S.*saprophyticus* (18.33%), others were *S. simulans* (10%), *S. xylosus* (10%), *S. chromogenes* (15%) and *S. schleiferi* (1.67%).

Staph. epidermidis has more frequency 68.5% than other CONS bacteria, this results is similar to Gad *etal.*, (2009) who show *S. epidermidis* represented 12.3% while *S. aureus* represented 6.3%, also it is fully CONS is fully consistent with the results of Arciola *etal.*, (2001) who explained that 60 strains of *S.epidermidis* were isolated from infections associated with the vascular catheter and 10 strains of *S.epidermidis* were isolated from the skin and mucous membranes of healthy subjects.

Table 4: Sugar fermentation for Coagulase negative Staphylococci bacterial strain.

CONS BACTERIAL STRAIN	SUGAR FERMENTATION								
	Glucose	***Fructose***	***Sucrose***	***Maltose***	***Lactose***	***Xylose***	***Mannose***	***sorbitol***	***Mlebiose***
Staph.epidermidis	+	+	+	+	+	-	+	-	-
S.saprophyticus	+	+	+	+	+	-	+	+	+
S.lentus	+	+	+	-	+	+	+	+	+
S.heamolyticus	+	+	+	+	+	-	-	-	-
S.hominis	+	+	+	+	+	-	-	-	-

Note: + : positive reaction; - : negative reaction.

Table 4 showed the *staph.epidermidis* isolated were none fermenting the xylose, sorbitole, mlebiose while fermented many sugar as positive results for glucose, fructose, sucrose, maltose, lactose and mannose, this results in agreement with [Ali *etal.*, 2009].

Table 5: Virulence factors which produce by Coagulase negative Staphylococci.

CONS BCACTERIAL ISOLATES	VIRULENCE FACTOR			
	DNase	**Urease**	**Lipase**	**Lecithinase**
Staph.epidermidis	-	+	+	-
Staph.saprophyticus	-	-	+	+
Staph.lentus	-	+	-	-
Staph.heamolyticus	-	-	-	-
Staph.hominis	-	+	-	-

Results in table 5 showed all *S.epidermidis* isolated were positive for Urease and lipase while negative for DNase and Lecithinase. *Staphylococci* release a large number of enzymes. Cunha *etal.*, showed of all CONS samples isolated produced Lipase (17.1%), lecithinase (3.4%), DNAse (15.4%), thermonuclease (7.7%) and enterotoxin (37.6%).

In the present investigation, except for S. *epidermidis* and *S. saprophyticus*, all species non produced lipase as well as DNase, Lipases play important role in persistence of bacteria by providing a source of energy or by facilitating adherence [Gribbon *etal.*, *1*993] as well as the lipases can be contribute to virulence by enabling bacteria to persist in the fatty secretions of

the human skin and also interfering with phagocytosis, Otto (2004) study finding the lipase of *S. epidermidis* can bind to collagen might constitute a novel role for lipase in virulence , while Urease was produced by 18.1% of CNS isolates[Alkhafaje, 2011].

Results of Longauerova (2006) study showed urease production by *S.simulans*, *S. capitis*, *S. hominis*, *S. warneri* and *S. caprae*, urease which clearly functions as important virulence factor, these findings are similar to those reported by Nataro *etal.*, (1994) and suggest that the infections caused by these microorganisms do not only depend on virulence factors but also on the conditions that predispose the host to infection, including factors innate and the use of invasive procedures.

Vuong (2000) study showed the S.epidermidis and other CONS are caused infected, it has been shown that extracellular products like protease, DNase, lipase, hemolysis and other exoenzymes may be responsible for tissue degradation and spreading of an infection caused by these bacteria.

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