EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 4
Issue: 12
01-08
Year: 2017

COLLECTION, ISOLATION AND IDENTIFICATION OF PATHOGENIC BACTERIA FROM BLOOD CLINICAL SPECIMENS IN BAGHDAD

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Article Received on 25/09/2017 **Article Revised on 15/10/2017** **Article Accepted on 05/11/2017**

ABSTRACT
One hundred and five of clinical sample were collected from Central Teaching Hospital of paediatric and Medical city / Educational laboratory, from patients (Male, Female and infant) of age range between(≤ 1 day-70 year)during the period from17/10/2016 to 23/1/2017. Out of one hundred and five clinical samples were screened, only seventy isolates were collected in 17 identified as *Staphylococcus aureus*, 12 identified as *Klebsiella pneumonia* depending on cultural, microscopical and biochemical characteristics. The remaining isolates were identified as *Staphylococcus* spp., *E.coli* and *Acinetobacter baumannii*, *pseudomonas aeruginosa*, *Enterobacter* spp and *Proteus* spp. Regarding to the patient gender, it was found that infants had a tendency to get infected more than males and female when 30 (28.57 %) of patients were males, 33 (31.42 %) females and 42 (40%) infant. Moreover, the age group ≤ 1year were most subjected to the infection of bloodstream infection.

KEYWORDS: Bacteriological, pathogenic, blood.

INTRODUCTION
Bloodstream infection is the most common cause of sepsis, so there are more than 45% of BSI are caused by single bacterial species, which can introduce in the blood, such opportunistic pathogens (e.g., *Staphylococcus aureus, Escherichia coli, Acinetobacter* spp, *klebseilla pneumonia*), and fungi species (Wisplinghoff *et al*., 2004; Risan, 2016). *Staphylococcus aureus* have different virulence factors which give the bacteria the ability to invade the host, such as surface proteins that promote colonization and invasiveness (leukocidin, kinases, hyaluronidase) and surface factors that inhibit phagocytic engulfment (capsule, ProteinA). *Klebseilla pneumonia* have different virulence factors which gave the bacteria the ability to invade the host, such as capsular polysaccharide, lipopolysaccharide, serum resistance, siderophore production, fimbriae and other factors such as the production of urea and enterotoxin (Aher *et al*., 2012). The pathogenicity of *Staphylococcus aureus*, is related to production of wide variety of exoproteins, including alpha and beta haemolysins which contributes to its ability to cause diseases in humans (Dinges *et al*., 2000). Alpha-haemolysin or alpha toxin considered to be a main pathogenicity factor because of its haemolytic, dermonecrotic and neurotoxic effects. Additionally, beta-haemolysin contains sphingomyelinase that more active against sheep and bovine erythrocytes (De-Silva *et al*., 2005). Staphylococcal enterotoxin B (SEB) is one of the 20 exotoxin excreted by the *Staphylococcus aureus* bacterium, Staphylococcal enterotoxin B (SEB) is the toxin most commonly associated with classic food poisoning, It has also been demonstrated to cause a nonmenstrual toxic shock syndrome (TSS) (Hennekinne *et al*., 2012). Therefore this study was aimed to isolation and identification of pathogenic bacteria in blood.

MATERIALS AND METHODS
Collection of samples
One Hundred and five samples of blood were collected from patients suspected of having blood stream infection and certain clinical symptoms. These samples were collected from Medical city Hospital / educational laboratories and Central Teaching Hospital of pediatric in Baghdad. Samples were collected from different age groups and genders from 17/10/2016 to 23/1/2017.

Blood samples
Blood is drawn from patients by using a syringe (5 ml). It is immediately transferred to a clean sterilized brain heart infusion broth tube, the blood is then allowed to clot for at least 10 to 15 minutes at room temperature, then kept in an incubator for 18 hours for further laboratory investigations (Tille *et al*., 2013).

Blood Culture
The blood specimens were inoculated on blood agar, McConkey agar and chocolate agar plates by direct

streaking method using a loop to deliver a loopful of the blood specimens. After incubation overnight at 37°C the bacterial growth is examined, if there were no growth, the plates were re-incubated for another 24 hours before they were considered as a negative culture (Novak-Weekley and Dunne, 2016).

Isolation and identification of bacteria

Bacteria have been isolated from pure colonies and cultured on blood, McConkey, and chocolate agar then isolated bacteria were examined microscopically by using Grams stain technique for referred to as Gram-positive or Gram-negative bacteria. The identification tests include cultural, morphology, and physiological characteristics of each bacterial isolates were done (Brown, 2005).

Identification of Morphological characteristics

Colonies of the bacterial isolates that cultured on blood agar and MacConky media were described according to their shapes, color, diameter, odor, and other characteristics (Macfaddin, 2000).

Microscopic Examination

The microscopic examination includes two procedures, gram stain, and capsule stain, according to (Atlas *et al*., 1995).

Biochemical tests

The following biochemical tests were performed for the identification of bacteria. These tests were carried out according to (Forbes *et al*., 2002) includes (Catalase test, Blood hemolysis test, Oxidase test and Indole test) and according to (Atlas, 2010) of Citrate utilization test and Urease production test.

Culturing on Eosin methylene blue (EMB) agar:

EMB, a differential medium used to distinguish *E. coli* isolates from others; bacterial isolates were cultured on this medium and incubated at 37°C for 24 hours. *E. coli* bacteria that grow on this medium gave a distinctive green metallic sheen indicate that the inoculated isolate belonged to *E. coli* (Atlas, 2010).

Mannitol fermentation: (Collee *et al*., 1996)

Mannitol semisolid agar medium was inoculated and incubated at 37 C˚ for 24 hrs. The changing of medium color to yellow indicates positive results for mannitol fermentation. This test is specific for *Staphylococcus aureus.*

RESULTS AND DISCUSSION

Isolation of bacteria

Blood samples from a total of 105 clinical different blood samples were collectedfrom Central Teaching Hospital of paediatric and Medical city / Educational laboratory, In Baghdad /Iraq. Table (1) samples were collected from different age groups and gender during the period from 17/10/2016 to 23/1/2017. Seventy (66.6 %) were clinical blood positive samples, while the rest (35) were negative blood samples (33.3 %).The relationship of BSI with the age of patients was investigated in this study and the patients were grouped into three categories according to their age as shown in table (1).

Table (1): Total number of samples used for the isolation of bacteria.

Clinical sample	Positive (growth)	Negative (no growth)
(105)	70	35
Percentage	66.66%	33.33%

Incidence of blood stream infection (BSI)

Blood samples from a total of 105 clinical different blood samples were collected, their ages ranging from(infant: 1 day - 12 month), (adult: 19 - 75 years). The results have showed that 70 (66.6%) of blood samples contained heavy bacterial growth while 35 (33.3%) of samples had no bacterial growth as demonstrated in table (2). This study agrees with cases in Pakistan (Latif and Ahmed, 2017) when they reported that incidence of BSI in patient were 97.6% while disagreement in India Waghmare *et al*., (2015) when they reported that incidence of BSI in patients were 18.6%.

Table (2): Distribution of incidence of BSI in relation to age of patients

Age group (Specimen)	(19-75) years		(1 day- 12 month)
	Male	Female	Infant
105	30	33	42
Percentage	28.57%	31.42%	40%

Identification of bacterial isolates

Several morphological, physiological and biochemical tests were made to identify bacterial isolates. Seventeen isolates were obtained from one hundred and five samples. Results showed that *Klebsiella* spp. constitute 17.1% (12 isolates), and identified as *K. pneumoniae*, *Staphylococcus* spp. Constitute 15.7% (11 isolates), *Staphylococcus aureus* constitute 24.2% (17 isolates). The other bacterial isolates were constituted *Escherichia coli* 14.2% (10 isolates), *Acinetobacter baumannii* 14.28% (10 isolates), *Proteus* spp. 2.82% (2 isolates), *pseudomonas aeruginosa* 7.14% (5 isolates), and *Enterobacter*spp. 4.28% (3 isolates). Figure (1) illustrates the percentages of each bacterial species found in the collected samples.

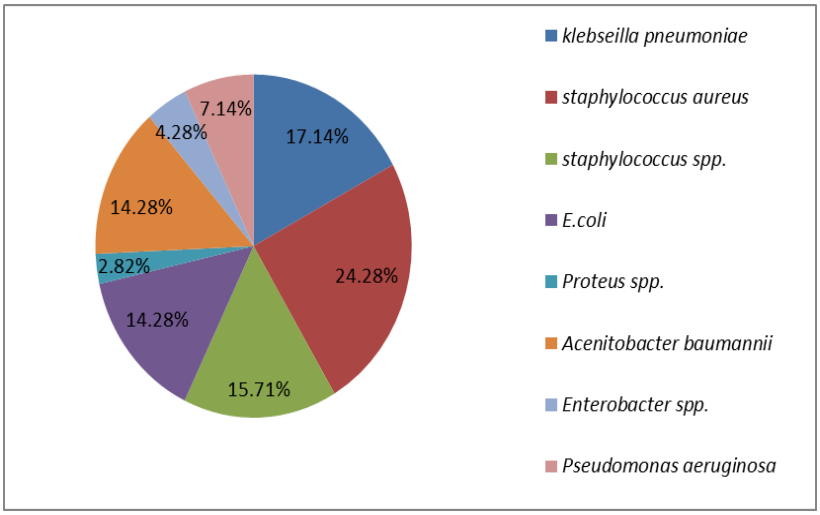
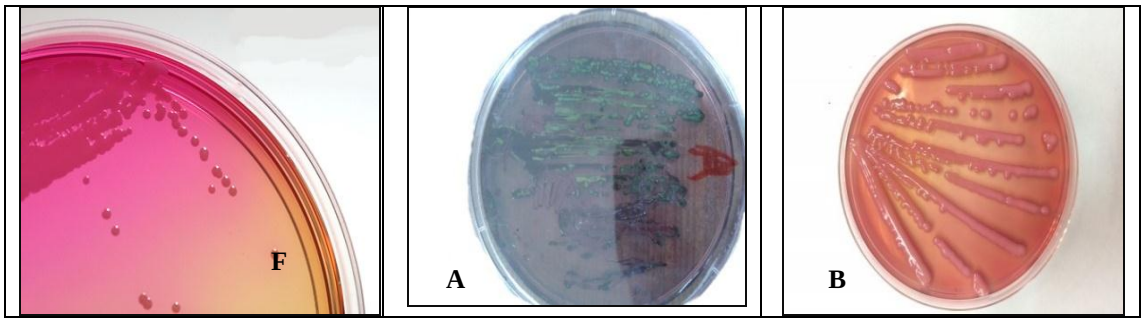


Figure (1): Bacterial isolates obtained from blood samples.

Bacterial isolates were identified according to their cultural, microscopical and biochemical characteristics that were in agreement with (Holt *et al.*, 1994; Atlas *et al*., 1995; Collee *et al*., 1996).

Colony morphology

First identification of bacterial isolates were done after incubated aerobically on MacConkey agar, blood agar and EMB agar plates and anaerobically on chocolate agar plates at 37 C^{o} for 24-48 hrs (figure 2- A-H). On MacConkey agar, *Klebsiella pneumoniae* are gram negative colonies which are lactose fermentingcolonies and gave pink color, regular edge, round; mucoid texture with large size. The first is characterized by producing pink colonies due to the conversion of neutral red indicator dye when it is below pH 6.8. Adversely, the Non-lactose bacterial growth appears colorless or transparent (Holt *et al.*, 1994). *Staphylococcus aureus* are gram positive colonies which are mannitol fermenting, about 1–2 mm in diameter and appears as grape-like clusters when viewed through a microscope, and has large, round, golden-yellow colonies. *Staphylococcus aureus* produces yellow colonies with yellow zones, whereas other coagulase-negative staphylococci produce small pink or red colonies with no colour change to the medium. *Escherichia coli* are gram negative, road shape, coliform bacterium. On MacConkey Agar grown for 24 hrs at 37 degrees, *E. coli* demonstrates strong lactose fermentation and gave bright pink halo, bile precipitant around the colonies, and pink colony growth, while on EMB agar which is selective media for *E.coli* which gave a distinctive metallic green sheen (due to the metachromatic properties of the dyes *E. coli* movement using flagella, and strong acid end-products of fermentation) (kim *et al*., 2002). *Pseudomonas aeruginosa* are gram negative, road shaped bacterium, with large colonies, irregular surface, yellow green colour, non-lactose fermenter whichproduces colonies with a characteristic "grape-like" or "fresh-tortilla" odour on bacteriological media (Hoiby *et al*., 2010). *Proteus* spp are gram negative bacilli, road shaped, large, circular grey, and smooth colonies. On blood agar shows swarming effect on the plate as a consequence of the organism motility activity, *Proteus* species do not usually ferment lactose, but have shown to be capable lactose fermenters depending on the species, give out an odour described as fishy (Drzewiecka, 2016). *Acinetobacter baumannii* is a typically short, almost round, rod-shaped (coccobacilli) gram-negative bacterium which grows well on MacConkey agar (without salt). *Enterobacter spp* is gram negative,colonial morphology ranging from smooth, irregularly round to rough. *Enterobacter spp*. grows rapidly on blood agar medium and MacConkey agar which large lactose-fermenting. In general, the strains from environmental sources grow better at 20-30 degrees, whereas strains from clinical sources grow better at 37C^{o}. "cauliflower" type colonies. Anaerogenic strains often exhibit yellow pigmented colonies (Holt *et al*., 1994).



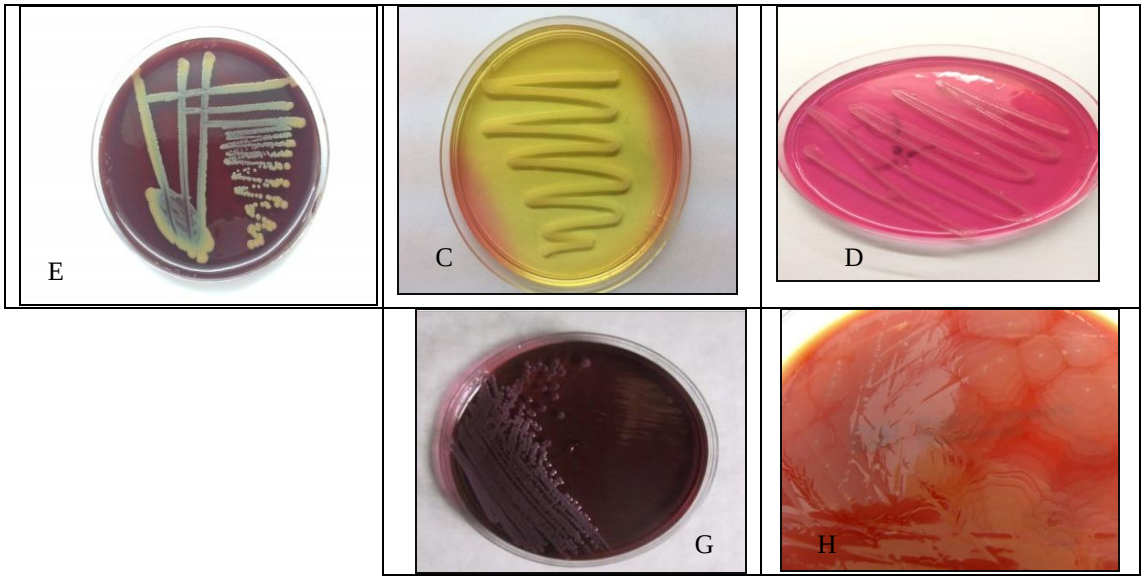


Figure (2): *E.coli* on EMB agar (A), Mucoid colonies of *Klebsiella pneumoniae* on MacConkey agar (B), Mannitol fermenting *Staphylococcus aureus* on Mannitol salt agar (C), Non-mannitol fermenting *staphylococcus epidermis* (D), Yellow green colour of *Pseudomonas aeruginosa* (E), *Acinetobacter baumannii* on MacConkey agar (F), *Enterobacter spp*. on blood agar (G), Swarming *Proteus spp*. on blood agar (H).

Biochemical Tests

The biochemical tests were used for further identification of bacterial isolates. Table (3) showed that all isolates of *Klebsiella*, *Staphylococcus*, *Proteus*, *pseudomonas*, and *Enterobacter* were negative result for indole while positive result for *E. coli*. In the indole test (Figure 3), ability to hydrolyze tryptophan to indole is a characteristic of certain enteric bacteria possessing the enzyme tryptophanase, an enzyme that decomposes amino acidtryptophan to indole, pyruvic acid and water. Indole negative bacteria was not produced tryptophanase, so that when Kovac's reagent was added to a broth free of indole, a red ring will not be formed at the top of the broth (Collee *et al.*, 1996).

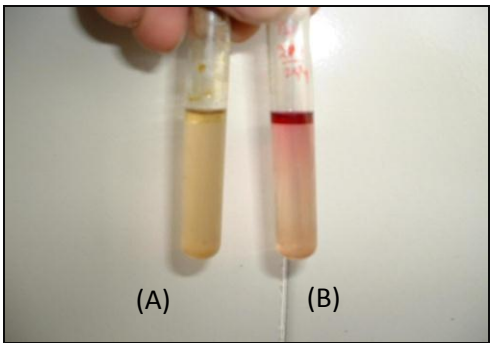


Figure (3): Indole test, negative result (A), positive result (B).

Utilization of citrate is one of several important physiological test used to diagnose members of all *Enterobacteriaceae* except *E.coli* which negative for citrate, while *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are positive for citrate. Citrate in simmon citrate medium is important to detect weather the bacteria isolates able to grow on it as a unique carbon and energy source. In addition, Simmon's medium also contains bromothymol blue as a pH indicator. *Klebsiella* is produced CO2, it reacts with components of the medium to produce an alkaline compound, the alkaline pH turns the pH indicator (bromthymol blue) from green to blue, reflecting it as positive citrate test (Macfaddin, 2000).

In Kligler Iron Agar (KIA) test, it differentiates the genera of *Enterobacteriaceae* from each other based on their carbohydrate fermentation patterns and H2S production. KIA slants contain 1 % lactose and 1 % glucose. The pH indicator (phenol red) changed the medium color from orange-red to yellow in the presence of acids. KIA also contains sodium thiosulfate, a substrate for H2S production, and ferrous sulfate that produces black precipitate to differentiate H2S producing bacteria from others.

Results Table (3) showed that *Klebsiella*, isolates turned the color of both the slant and butt, which produced acidic slant (yellow) and acid butt (yellow) accompanied by gas production (bubbles formation), but without black precipitate formation, which indicates that lactose and glucose fermentation had occurred and no H2S was produced. These results agreed with those declared by Garrity (2005). *E.coli* isolates turned the color of both the slant and butt, which produced acidic slant (yellow) and acid butt (yellow) accompanied by CO_2 production but without black precipitate formation, which indicates that lactose and glucose fermentation had occurred and no H2S was produced, These results agreed with those declared by (Penalver *et al*., 2005).

Proteus spp isolates turned the color of both the slant and butt, which produce acidic butt (yellow) and alkaline slant (red) accompanied by H_2S production (black precipitant) that indicates of glucose fermenting and non-

lactose fermenting,These results agreed with those declared by (Saadabi *et al*., 2010). *Acinetobacter baumannii* isolates turned the colour of slant to alkaline butNo change bottom, No gas, No H_2S production that indicate non-lactose fermenting, These results agreed with those declared by (Hussein *et al*., 2013). *Pseudomonas aeruginosa* isolates turned the colour of slant and butt to alkaline without production of H_2S and gas that indicate non-lactose fermenting, these results agreed with those declared by (Tunç and Olgun, 2006).

Table (3): Cultural, Microscopically, Physiological and Biochemical characteristics of different bacterial isolates.

No.	Test \ Isolates		*K. pneumoniae*	*S. aureus*	*E. coli*	*A. baumannii*	*Proteus*	*P. aeruginosa*	*Enterobacter*
1	Cell shape		Bacilli	Cocci	Bacilli	Coco-bacilli	Bacilli	Bacilli	Bacilli
2	MacConkey agar		LF	LNF	LF	LNF	LNF	LNF	LNF
3	Gram stain		-	+	-	-	-	-	-
4	Capsule stain		+	-	-	-	-	-	-
5	Motility		-	-	V	-	V	+	V
6	Urease		+	+	-	-	+	-	-
7	Indole		-	-	+	-	-	-	-
8	Citrate utilization		+	+	-	+	V	+	V
9	Kliglar iron agar (KIA)	H_2S	-	-	-	-	+	-	-
		CO_2	+	-	+	-	ND	+	-
		Acid	A/A	ND	A/A	K/K	A/K	K/K	A/A
10	Oxidase		-	-	-	-	-	+	-
11	Catalase		V	+	V	+	V	+	+
12	Mannitol fermenter		V	+	V	-	-	V	V
13	Coagulase		ND	+	-	ND	ND	-	-

(+) positive result,(–) negative result, (ND) Not determined, (K) alkaline, (A) acid, (V) variable result, (LF) Lactose ferment, (LNF) Lactose Non-ferment.

Whereas urease test, figure (4), were positive for *Klebsiella*, *Staphylococcus* and negative for *Enterobacter*, *proteus*, *E.coli*, *pseudomonas* and *Acinitobacter* (Forbes *et al.*, 2002). Urease enzyme catalyzes the breakdown of urea, and the bacteria that can produce this enzyme is able to detoxify the waste products and to drive metabolic energy from its utilization which change the medium color from yellow to purple-pink, indicating urease positive test. *Klebsiella* can produce urease enzyme and gives urease positive test (Atlas *et al*., 1995).

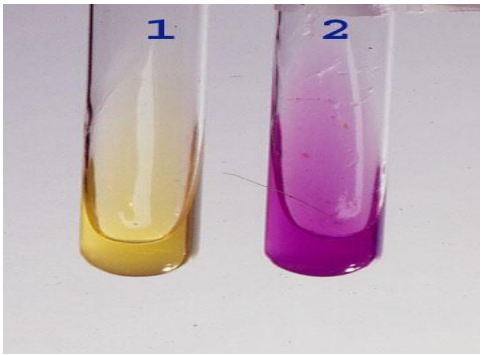


Figure (4): urease test, positive result (2), and negative result (1).

In the motility test, *Klebsiella* isolates were non-motile. The movement of the growth away from the stab line or a hazy appearance through the semisolid medium indicates that the bacteria are motile. But the linear growth means negative result a property which *Klebsiella* is characterized by Gwendolyn (1988). While *Acinetobacter baumannii*isolates were non-motile, these results agreed with those declared by (Hussein *et al*., 2013).

Moreover *E.coli*, *Pseudomonas aeruginosa*, *Enterobacter* spp, and *Proteus* spp isolates virable motile as expiated in figure (5) (O'toole and Kolter, 1998; RÖmling, 2005; Pomorski *et al*., 2007; Berg, 2008).

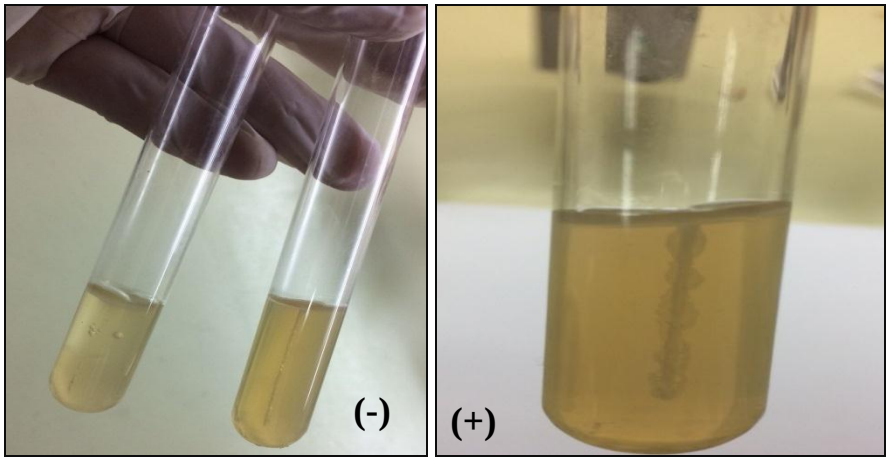


Figure (5): motility test, (-) negative result, (+) positive result.

Another hand in oxidase test, figure (6), *Klebsiella*, *E.coli*, *proteus*, *Enterobacter* isolates were oxidase negative and catalase positive or negative result (Bernere and Farmer, 2005), where else *Pseudomonas aeruoginosa* isolates were oxidase positive and catalase positive (PHE, 2015), while *Staphylococcus aureus* isolates were oxidase negative and catalase positive (Orwin *et al*., 2003), and finally *Acinetobacter baumannii* isolates were oxidase negative and catalase positive (Doughari *et al*., 2011). The coagulase test is specific to differentiate *Staphylococcus aureus* from other species and genera which is positive (Kateete *et al*., 2010).

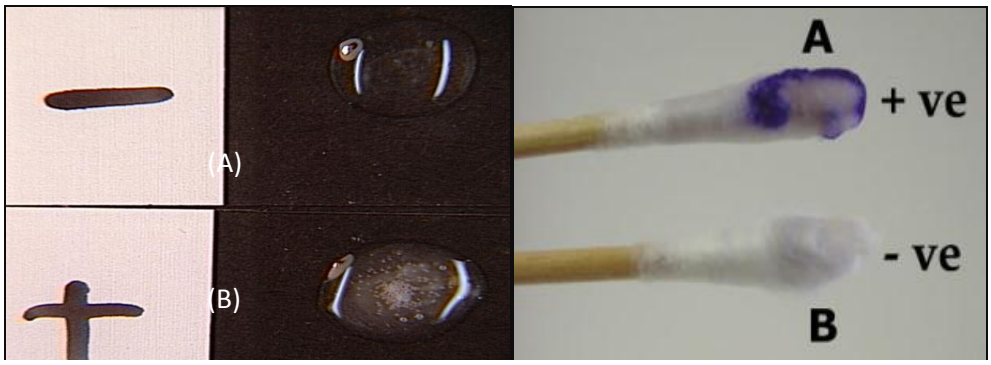


Figure (6): Left (coagulase test (A) positive result, (B) negative result) Right (oxidase test (A) positive result, (B) negative result).

Distribution of bacterial isolates according to the samples

Results presented in table (4) show that out of 105 clinical samples, 17 (15.7%) *Staphylococcus aureus*, 12 (17.1%) *Klebsiella pneumoniae*, 11 (24.2%) *Staphylococcus* spp., 10 (14.2%) *E. coli*, 10 (14.2%) *Acinetobacter baumannii*, 5 (7.14%) *pseudomonas aeruginosa*, 3 (4.28%) *Enterobacter spp*. and 2 (2.82%) *Proteus spp*. isolates were recovered.

Table (4): Types and numbers of bacterial isolates obtained from samples.

No.	Bacterial isolates	Number of isolates
1	*Klebsiella pneumoniae*	12 (17.14%)
2	*Staphylococuus aurues*	17 (24.28%)
3	*Stapylococcus spp*	11 (15.71%)
4	*Escherichia coli*	10 (14.28%)
5	*Proteus spp*	2 (2.82%)
6	*Acinetobacter baumannii*	10 (14.28%)
7	*Pseudomonas aeruginosa*	5 (7.14%)
8	*Enterobacter spp*	3 (4.28%)
	Total	70 (66.66%)

Results revealed that *S. aureus* and *K. pneumoniae* (17, 12 isolates respectively) was the dominant among all other species of bacteria. This result was in agreement with the report documented by Waghmare *et al*., (2015)

and Karki (2010) whom found that those two species was the most frequently occurring among other species, when its account for 29 % of *Klebsiella pneumoniae* and 65% of *Staphylococcus aureus* isolated clinically.

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