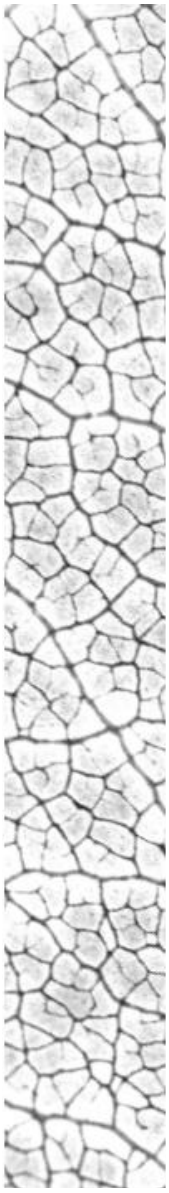

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Phenotypic detection and vancomycin MICs for methicillin resistance Staphylococcus aureus isolated from nebulizer

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Abstract
Nebulizer and other respiratory care devices became as reservoir of pathogens when colonized with microorganisms and play important role in transmitted from patient to an another, Methicillin resistance Staphylococcus aureus is one of the main pathogens caused nosocomial infection in particular hospital respiratory infections. during a 6- month period 52 swab samples were collected from contaminated nebulizer masks, all samples were cultivated on mannitol salt agar then chromogenic agar (CHROMagar™ Staphylococcus aureus and CHROMagar™ MRSA) were used for characterizing S. aureus and MRSA, cefoxitin disk diffusion method were also performed, vancomycin sensitivity by disk diffusion method and minimum inhibition concentration were evaluated using fluorescent microscope and subculture. 38.46% (20/52) were S. aureus identified on mannitol salt agar and on CHROMagar™ Staphylococcus aureus detected 19/52 (36.5%), 17 isolates of them were MRSA detected by CHROMagar™ MRSA and cefoxitin disk diffusion method, the vancomycin MICs value were 1.25µg/ ml of 64.71% and 1.75µg/ml of 35.29% . nebulizers applying by many different patients therefore can play a vital role in controlling on infection within a hospital, MRSA are associate with these infections detecting with significant numbers and resistant to many antibiotics, phenotypic detection like; chromogenic agars and cefoxitin deck diffusion method are highly recommended for rabid detection, vancomycin has high effective on MRSA and the best drug for treating bacterial resistance infections.

Keywords: nebulizer, MRSA, CHROMagar, cefoxitin disk diffusion method, vancomycin MIC

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INTRODUCTION

Nebulizer is an important respiratory car device uses for treating of Chronic Obstruction Pulmonary Disease (COPD), Asthma and other respiratory conditions (Jadhav et al. 2013). Respiratory care equipments which including nebulizers, humidifiers and ventilators involved in nosocomial respiratory infections when they are colonized by bacteria or fungi (Jadhav et al. 2013, Nitin and Hassani 2016, Sims et al., 2020), where many type of microorganisms have been isolated from nebulizers and humidifiers by Nitin and Hassani (2016) like gram negative bacilli (90.33%): Pseudomonas, Klebsiella, Burkholderia cepacia, Acinetobacter, and gram positive cocci (9.33%): coagulase negative Staphylococcus and Staphylococcus aureus . Rello et al. (1991) found 21.9 percent of patients who use mechanical ventilation developed a bacterial pneumonia and gram negative bacilli have been identified as a first pathogens (62.2 %) and the second one was Staphylococcus aureus (23.2%) with high incidence. S. aureus is significant pathogen for humans and responsible for numerous sever infections (Diaz et al. 2018), it is also considered one of the major cause of nosocomial infections (Jain et al. 2008), in particular Methicillin resistance S. aureus (MRSA) where it emanated as importance pathogen in the hospital infections over latest few years (Diaz et al. 2018, Jain et al. 2008, Wilcox et al.2019).

MRSA, generally like *S. aureus* and other bacteria, is transmitting via physical contact. This can happen through direct contact between the infected person and the uninfected person or via indirect contact by a vector, such as a health care workers, health care equipments or contaminated objects or surfaces (Zhang and Burbridge 2011).

Newly, hospital and community – acquired infections with *S. aureus* and antibiotics resistant isolates increasing (Jain et al. 2008, Rağbetli et al. 2016). The developing of resistance to plentiful antibiotics by *S. aureus* has been implicated by gene transfer horizontally

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of mobile genetic elements, resistance also can gain by mutations in genes chromosomes that modify the binding sites of drug on molecular targets and by rising endogenous efflux pumps expression, MRSA has *mecA* gene therefore resistance Methicillin and other β-lactam antibiotics (Foster 2017).

The recent identification ideal of *S. aureus* via chromogenic media are widely used compare with conventional media, the last need followed with biochemical tests (Bakr and Selim 2007, Gaillot et al. 2000), as well as recently, the Clinical and Laboratory Standards Institute M100-S23 (CLSI)) (2013) indicates that use the disk diffusion technique of cefoxitin, oxacillin and oxacillin screen test have been recommended as phenotypic method for MRSA detection. some studies have been declared low specificity and low sensitivity of oxacillin confronted with cefoxitin (Jain et al. 2008; Akpan and Udoh, 2017).

The present study has been focused on frequent of MRSA in contaminated nebulizers, although had significant number in many studies but had been a little concern separately. chromogenic agars and phenotypic tests have been used for characterizing. finally the MIC of vancomycin have been evaluated using fluorescent microscope and sub cultures.

MATERIALS AND METHODS

Samples Collection

Fifty two swab samples were collected from nebulizer's masks of patients were attended the emergency departments of many hospitals in Basrah city from October 2018 to March 2019. The samples were transported to research laboratory in Clinical and Laboratory Science Department – College of Pharmacy, brain heart infusion was used as transporter media.

Isolation and Identification

As an initial isolating step all samples were cultivated on selective and deferential media: mannitol salt agar(MSA) (BIOMARK- India) (Bakr and Selim 2007, Kateete et al. 2010) and chromogenic agar: CHROMagar™ *Staphylococcus aureus* (ChSA) (CHROMagar™ - Paris- France) and were incubated for 24 hr at $37C^0$ under aerobic conditions (Al-Tameemi 2018, Bakr and Selim 2007, Dawod et al. 2020). As a next step of identification the samples were streaked onto other deferential medium: chromogenic agar: CHROMagar™ MRSA (ChMRSA) (CHROM agar ™ - Paris- France) then were incubated at $37C^0$ aerobically for 24 hr (Hasan 2016, Micheel 2015).

All these media were prepared as manufacturer instructions. Gram stain and examination by microscope was included.

Antibiotic Sensitivity Assay

The sensitivity of isolates to cefoxitin (30µg) and vancomycin (30µg) were performed on Muller Hinton agar (MHA) (OXOID-England) using an agar disk diffusion method was described by CLSI M02-A12 (2015), the suspensions (0.5 McFarland) of bacterial isolates were spread on the seeded plates then the disks of antibiotics were applied on the inoculating agar and were incubated for 18 hr at 35 ^{0}C aerobically. The inhibition zones (IZ) results were read according CLSI M100-S23 guidelines (2013). cefoxitin disk diffusion method (cefoxitin DDM) was applied on all *S. aureus* isolates were identified by previous steps for confirming detecting of *mecA* gene according to CLSI M100-S23 (2013), while vancomycin was tested on MRSA isolates only to evaluated the sensitivity as first step.

Evaluation of Vancomycin MICs

Broth macro dilution test was used for determination of vancomycin sensitivity and MICs values, Muller Hinton broth (MHB) (Solucea- Netherland) was distributed in tubs after preparation and sterilization, 100µl was added from bacterial inoculum of 0.5 McFarland plus seeded amounts form stock solution of antibiotic were added using micropipette to prepare serial dilutions (0.125- 2.5 µg/ml) , extra two tubs were included : one was control positive (MHB with bacterial suspensions) and the other was control negative (only MHB) (CLSI M07-A10 2015).

Viability Tests

The viability of MRSA isolates was investigated via subculture on nutrient agar (OXOID- England) after 24hr (Kali et al. 2014). MIC was recorded as the lowest concentration was performed a significant decreasing in bacterial viability

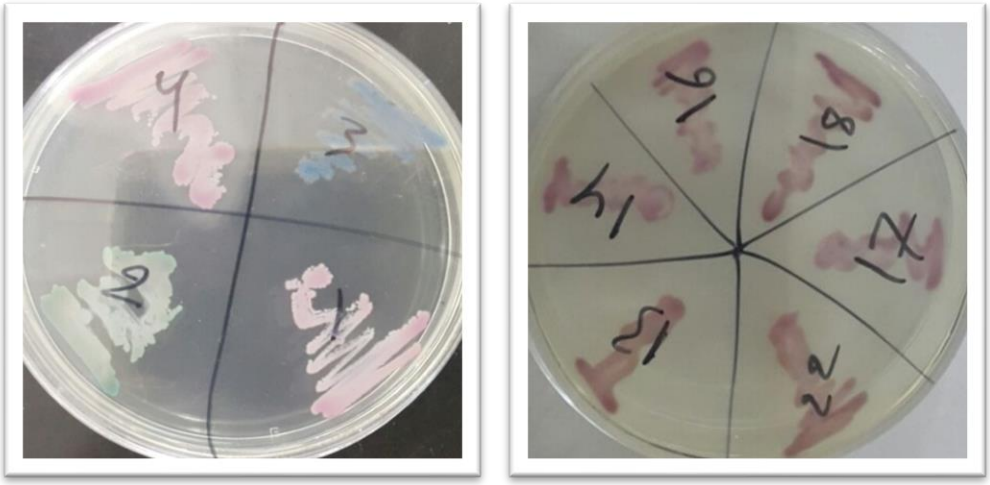
Viability of bacterial cells were also examined using fluorescent microscope according to Abdul Jabbar (2017) with little modification , after 24 hr of incubation, bacterial suspension were centrifuged at 7000 rpm for 3 min the supernatant was removed and the sediment was re-suspend with 2 ml of sterile distilled water, the samples were washed three times, 10 µl of bacterial suspension were applied on clean glass slide then were mixed with 25µl of Acridine orange dye that label live and dead bacterial cells with green and red color respectively.

The mixture was covered by clean slip and was left at room temperature for 15 min before adding oil emersion and screening by fluorescent microscope , this procedure was repeated for each tube, count of dead and live cells were performed using Image J program. The viability of bacterial cells = number of live cells / number of live cells +number of dead cell * 100.

RESULTS

S. aureus Identification

Twenty isolates 38.46 % out of fifty two samples of nebulizer's masks were identified on MSA as yellow and mannitol fermenting colonies and 19/52 isolates 36.5% were assessed as *S. aureus* on CHROMagar™ *S. aureus* (ChSA) which produced pink to mauve colonies



(a) (b)

Fig. 1. a- pink to mauve *S. aureus* colonies on ChSA , b- rose to mauve MRSA colonies on ChMRSA

Table 1. CHROM agar *S. aureus* contra mannitol salt agar

MSA	ChSA		Total
	positive	negative	
positive	19	1	20
negative	1	31	32
Total	20	32	52

MSA: mannitol salt agar, ChSA: CHROM agar *S. aureus*

Table 2. Phenotypic detection of *mecA* gene in *S. aureus*

Bacterial isolates	ChMRSA	Cefoxitin DDM
mecA gene positive	17	17
mecA gene negative	2	2
Total	19	19

ChMRSA: CHROMagar™ MRSA, DDM: disc diffusion method

as shown in **Fig. 1a**, on ChSA, only one yellow colony was false positive (didn't confirmed as *S. aureus*) these results were related with sensitivity 95% and specificity 96.8% of ChSA as seen in **Table 1**.

All these colonies gave Gram positive reaction.

Sensitivity=test $^{+}$ve / test $^{+}$ve + test $^{-}$ve * 100 = 19/1+1*100=95%

Specificity=test $^{-}$ve / test $^{-}$ve + test $^{+}$ve * 100 = 31/31+1*100=96.8%

MRSA Identification

Out of nineteen isolates previously isolated on ChSA 17 isolates 89.47 % and 32.69% out of total specimens were assessed on CHROMagar™ MRSA (ChMRSA) as MRSA, they appeared rose to mauve colonies in color as shown in **Fig. 1b**.

The cefoxitin DDM showed that 17/19 *S. aureus* isolates were positive to *mecA* gene, where its resistant to cefoxitin , all of them gave inhibition zones (IZ) < 20 mm and the rest 2 isolates were negative and sensitive to cefoxitin the IZ were 22 mm and 23 mm assessed as MSSA (**Table 2**).

The sensitivity of cefoxitin DD M and ChMRSA reached to 100%.

Vancomycin Activity

The viability test by subcultures and fluorescent microscope images illustrated gradually decreasing of

Table 3. Viability of MRSA isolate group A & B according to subcultures

Concentrations µg/mL	Sub culture GA	Sub culture GB
0.125	++	++
0.25	++	++
0.5	++	++
0.75	++	++
1.0	+	++
1.25	+ -	++
1.5	-	+
1.75	-	+-
2	-	-
2.25	-	-
2.5	-	-

GA: group A, GB: group B,++: heavy bacterial growth, +: moderate bacterial growth, +-: ≤10 bacterial colonies, - : no growth

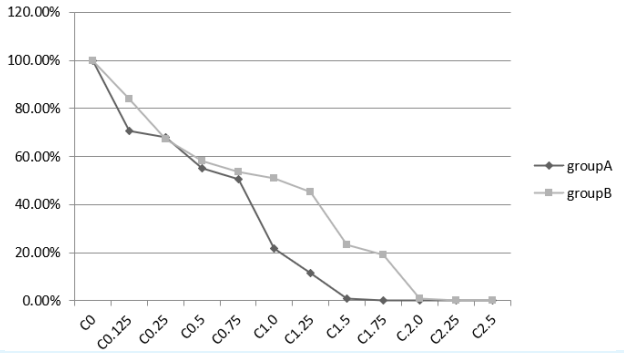
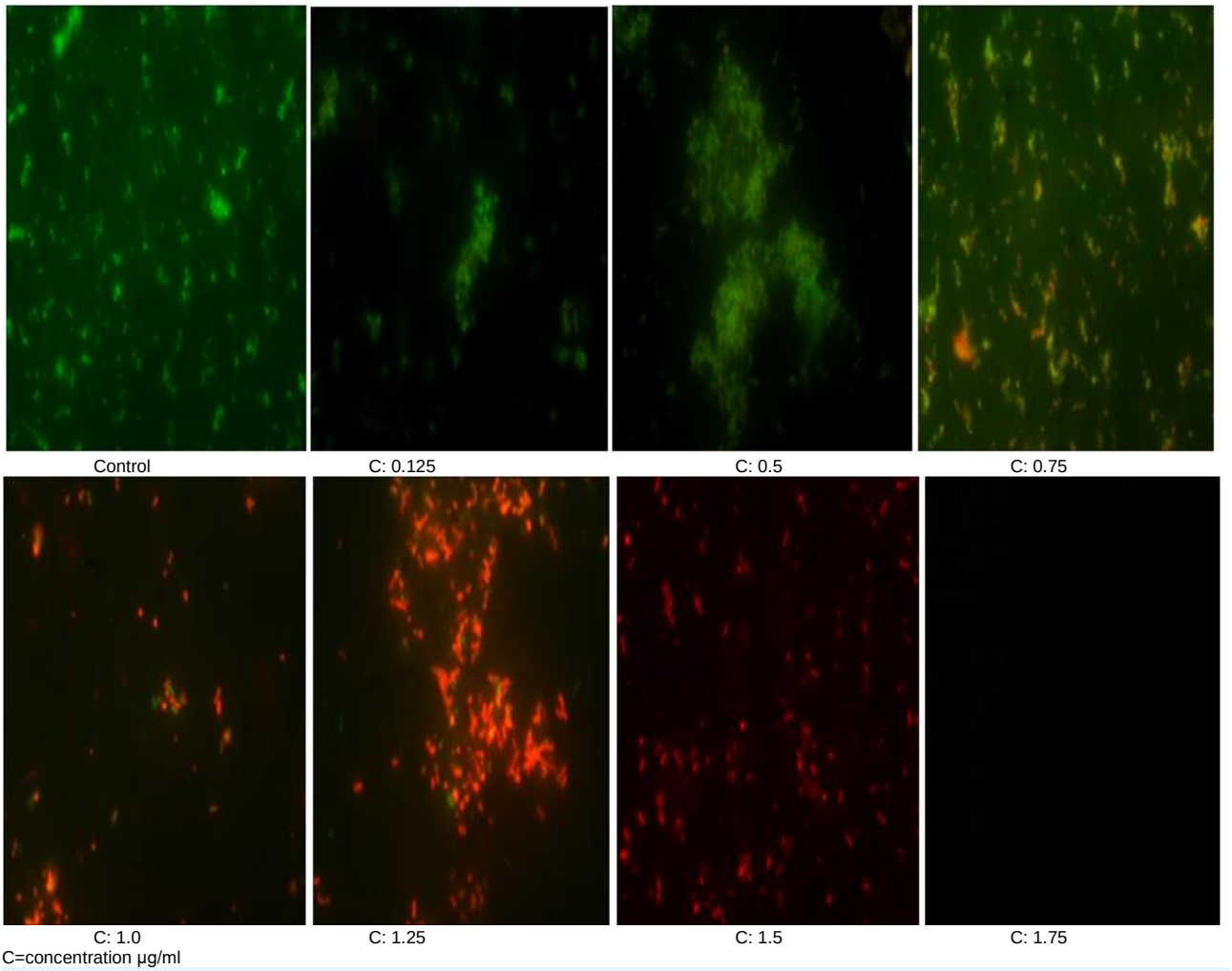


Fig. 2. Viability of MRSA isolates groups A& B according to fluorescent microscope images

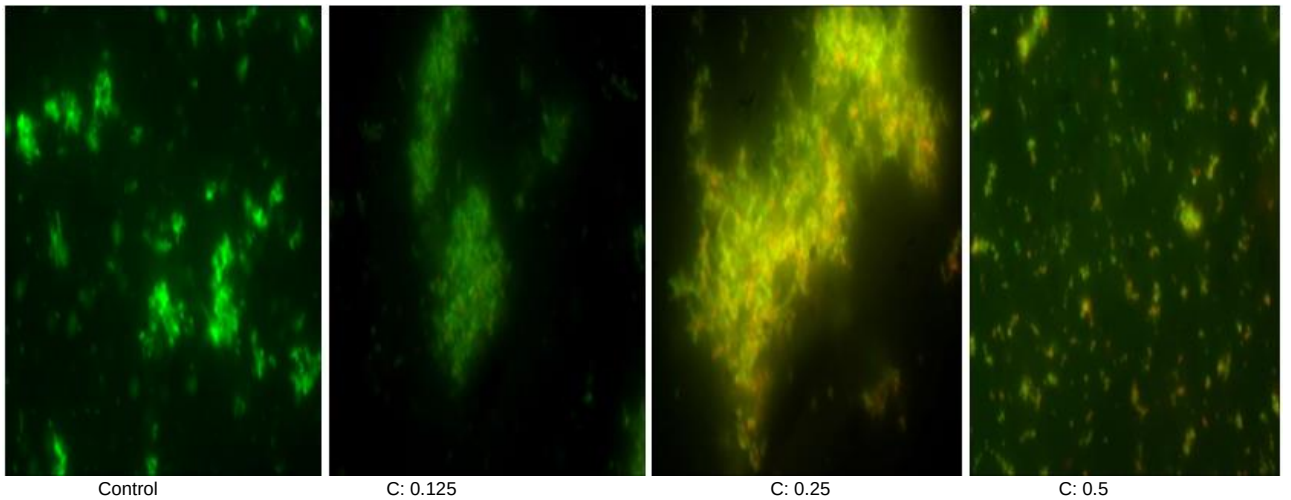
cells viability contra with increasing vancomycin concentrations as noticed in **Table 3** and **Figs. 2**-**4**. These results assessed the group A of MRSA were higher sensitive to vancomycin concentration than group B, where 30 % of group A bacterial cells have been inhibited by concentration 0.125µg/ml (**Fig. 2**) and the MIC value was 1.25 µg/ml (**Figs. 2**,**3** and **Tables 3**,**4**), compared with MRSA group B had MIC 1.75 µg/ml (**Figs. 2**, **4** and **Tables 3**, **4**).

The antimicrobial activity findings of vancomycin disk diffusion method against MRSA was 11 isolates (group



C=concentration µg/ml

Fig. 3. Activity of vancomycin concentration on viability of MRSA group A by fluorescent microscope



C=concentration µg/ml

Fig. 4. Activity of vancomycin concentration on viability of MRSA group B by fluorescent microscope

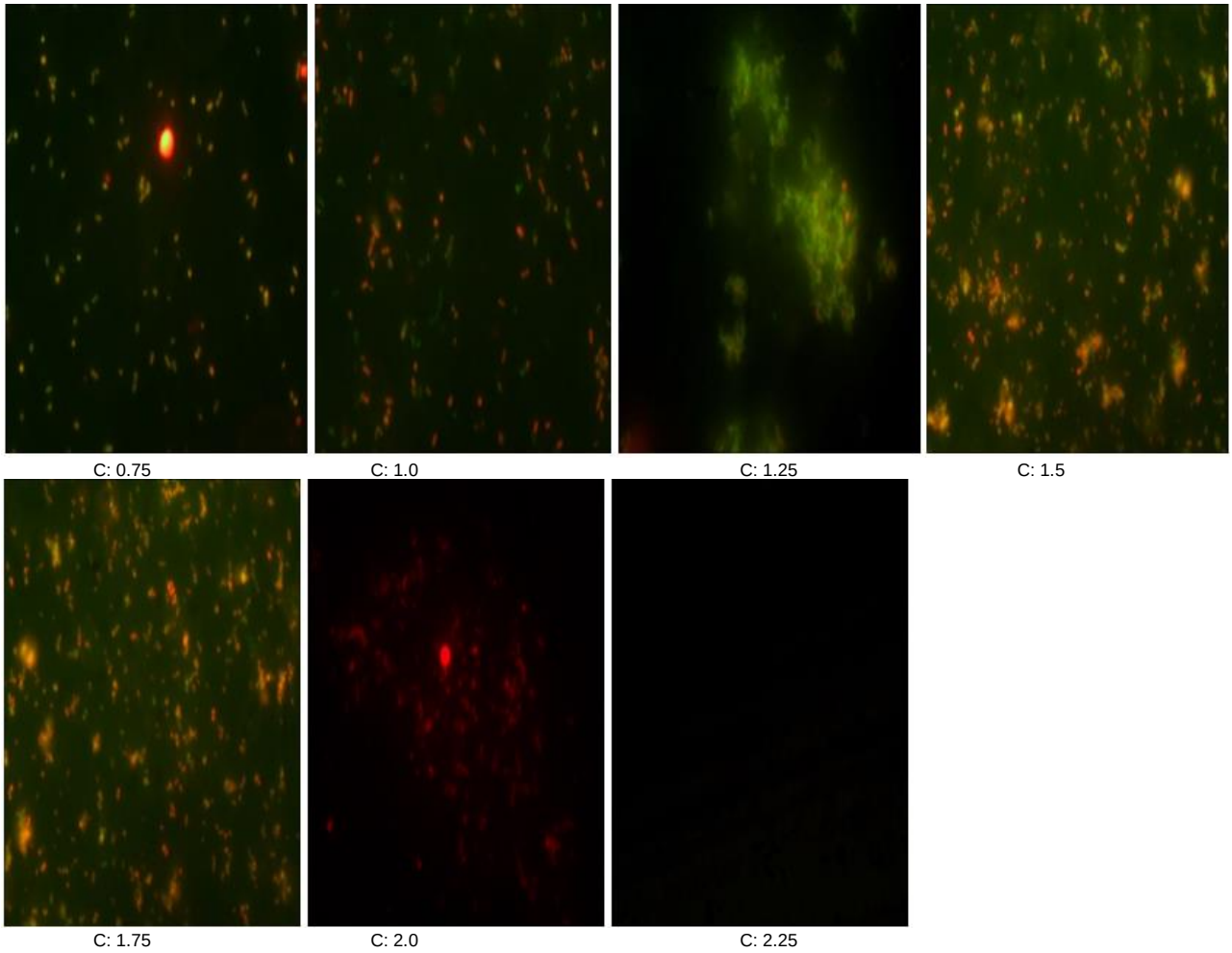


Fig. 4 (continued). Activity of vancomycin concentration on viability of MRSA group B by fluorescent microscope

Table 4. Vancomycin sensitivity of MRSA isolates

NO. isolates	percentage	Vancomycin DDM IZ	MIC values
GA 11	64.71%	18mm	1.25 µg/mL
GB 6	35.29%	15mm	1.75 µg/mL

GA: MRSA group A, GB: MRSA group B, DDM: disc diffusion method, IZ: inhibition zone

A) out of 17 MRSA gave IZ =18mm and the reminder 6 gave 15mm (group B) as shown in **Table 4**.

DISCUSSION

Nebulizer is one of the important respiratory care devices and can plays essential role in transmission the infections among the employing patients when be contaminating with microorganisms, respiratory tract infections are the most common type of nosocomial infection (Jadhav et al. 2013, Nitin and Hassani 2016). Approximately 40% of nosocomial infection is nosocomial pneumonia (Jadhav et al. 2013). *S. aureus.* is one of main pathogens of nosocomial disease and the major clinical problem is outbreak of MRSA (Foster 2017). Ewing et al. (1999) classified *streptococcus pneumonia and staphylococcus aureus* as potentially pathogenic microorganisms PPMs causing respiratory infections. Rello and Diaz (2003) demonstrated that 80% of total hospital pneumonia was ventilation –associated pneumonia and MRSA responsible for 8.2%.

In present study from 52 samples of contaminated nebulizer's masks; *S. aureus* has n= 20 (38.46%) and 19 (36.5%) were differentiated on MSA and ChSA respectively, MSA is selective and differential medium has widely uses in vitro to isolation and identification of *S. aureus* with high sensitivity 94% (Kateete et al. 2010), several authors were founded the sensitivity of MSA was raised with increasing the incubation period where increased from 71% to 96% after 24 hr and 48 hr of incubation respectively (D'Souza and Baron 2005), in contrast low sensitivity even after 48 hr incubation 73.49 % (Bakr and Selim 2007). There are variation of sensitivity rate of MSA therefore we need additional tests to confirm the identification.

Identifying *S. aureus* on ChSA was recorded 36.5 % out of 52 samples, our results were proved the potential of ChSA for isolating and identifying *S. aureus* from mixed clinical samples with sensitivity reached to 95% and specificity 96.8% near to our results, many studies

indicated the sensitivity of ChSA had 98.79% ,95.5% and 98.6%, respectively (Bakr and Selim 2007,Gaillot et al. 2000, Kluytmans et al. 2002).

Percentage of *S. aureus* were isolated from contaminating nebulizer in present study was high compare with other studies were recorded *S*. *aureus* 7.2%, 11% and 3.2% (Al-Tameemi 2018, Dawod et al. 2020, Nitin and Hassani 2016). These results may have indicated for outbreak of *S. aureus* as PPMs which is opportunistic pathogen and may because transmitted from person to another. Contaminated respiratory care equipments perhaps involved with nosocomial infections by 2 ways; firstly: Equipments may become as a reservoirs for microorganisms, when the fluid of devices such as humidifiers and nebulizers and become contaminated by bacteria and fungi which may be capable of multiplying in water. The pathogens may then spread to the patients by aerosolization in the room. Secondly, the delivery or the direct investiture of pathogens to the airways may results from contaminated equipments, if the equipments linked directly to the ventilator system or if contaminated drug is aerosolized or instilled. Several equipments such as nebulizers chambers and oxygen masks may be transported from patient to the other many times daily and they may be rarely cleaned daily (Al-Tameemi 2018, Jadhav et al. 2013, Nitin and Hassani 2016).

ChMRSA characterized 17 isolates 89.47% out of 19 *S. aureus* isolates were identified previously on ChSA , ChMRSA is deferential and selective medium developed for detecting MRSA in clinical samples such respiratory samples with high sensitivity and specificity (Hasan 2016, Micheel 2015), similar to these results; Dawod et al*.* (2020) and Al-Tameemi (2018) where detected 8 MRSA isolates of 9 *S. aureus* isolates .The high rate of appeared multi-resistance strains like MRSA due to antibiotics bad using ,taking antibiotics without caregiver following and directive medicine giving of antibiotics and granting antibiotics for a long period (Hasan 2016).

Regarding employing cefoxitin DDM in our study for detecting *mecA* gene as the sole maker has recommends by CLSI M100-S23 (2013). If IZ <22 mm that indicator of the isolate carry *mecA* gene and the isolate assesses as MRSA versus IZ ≥ 22 mm the isolate should be out MRSA, present funding; detecting *mecA* gene in 17/19 isolates and confirmed as MRSA because of the resistance to cefoxitin, where IZ ranging between 16-20mm only two isolates were false negative.

S. aureus resistance β-lactam antibiotics by production Beta-lactamase encoded by *mecA* gene, which inactivated penicillin either by hydrolysis of Beta-lactam ring or by association with penicillin binding protein 2^a ($PBP2^a$) (Duran et al. 2012).

The results of ChMRSA and cefoxitin DDM identification were full agree, 17 isolates were carried *mecA* gene detected at both tests. Recently there are more approach for using chromogenic agars such as CHROMagar *S. aureus*, CHROMagar MRSA, CHROM ID MRSA etc. these agars employ chromogenic substrate to characterize *S. aureus* from other causative agent and selective the growth of MRSA via antibiotics (Hasan 2016, Hedin and Fang 2005), 97.8% was the sensitivity of ChMRSA have been recorded by Kali et al. (2014), Hedin and Fang (2005) recorded sensitivity 95.4% after 24 hr and raised to 100 % after incubation for 48 hr .Using chromogenic agars reduce time , confirmatory tests that may high cost and intensive work (Bakr and Selim 2007, Gaillot et al. 2000, Hasan 2016). As well as cefoxitin DDM recommended by CLSI M100-S23 (2013), high sensitivity 97.3%, and specificity 100% has been recorded by Broekema et al. (2009), in study of Anand et al.(2009) the sensitivity and specificity reached to 100% and emphasized using the cefoxitin DDM as an alternative test to PCR because of this test absolutely accords with PCR technique for detection *mecA* gene in *S*. *aureus*. cefoxitin DDM has recommended at many recent studies compare with oxacillin because of complicated detection of oxacillin resistant strains showed resistance at deferent levels (Jain et al. 2008). However, the phenotypic detection of MRSA are affected by the conditions of culture , although the PCR technique deemed the gold stander for detecting *mecA* gene but limited using due to greater technical, cost constraints and poor resource in many hospitals and laboratories (Kali et al. 2014), otherwise the phenotypic methods proved its ability and have recommended from many studies for characterized *mecA* gene in *S. aureus*.

On the other hand the sensitivity test of vancomycin against MRSA isolates recorded IZ ≥ 15 mm ,according to American Society for Microbiology(ASM) all these isolates were 100% sensitive to vancomycin (Hudzicki 2009), while the CLSI M100-S23 (2013) illustrated the performing of MIC tests are necessary to determine the sensitivity of Staphylococci toward vancomycin, the disc test un sufficient to differentiate the sensitive isolates.

All MRSA isolates were 100% sensitive to vancomycin and the MIC values were 1.25 µg/ml for 64.71 % of MRSA isolates (GA) and 35.29 % of them (GB) had MIC = 1.75 µg/ml, CLSI M100-S23 (2013) demonstrate the isolate considered sensitive to vancomycin if had MIC ≤ 2 µg/ml.

These results met with finding of many studies; Prakash et al. (2008) evaluated the MIC of 101 MRSA isolates, the MIC value were 0.5µg/ml to 21 isolates, 1µg/ml to 77 isolates and 2µg/ml to 3 isolates. While the MRSA MIC of vancomycin extend from 0.125µg/ml to 1µg/ml (Kshetry et al. 2016). Resistance to vancomycin result from re-modeling of the cell wall to decrease access of drug to the lethal target that require more than six mutations in various genes (Foster 2017), therefore vancomycin still the best choices for most MRSA nosocomial infections (Diaz et al. 2018,Wilcox et al. 2019,Zhang and Burbridge 2011).

The detecting of MRSA accurately and rapidly play important role in therapy the infection correctly and controlling on mortality (Jain et al.2008, Kali et al. 2014, Wilcox et al. 2019), where the failure in treatment patients of *S. aureus* bacterimia increase the mortality whenever MRSA had high vancomycin MIC ≥ 1.5µ g/ml (Wilcox et al. 2019). As well as other studies have been reported failure of vancomycin treatment in particular MRSA which had vancomycin MIC ≥ 4µg/ml, the recent observations were asserted the increasing in values of vancomycin MIC against MRSA may be led to appearance full resistant isolates (Diaz et al. 2018).

On the other hand, MRSA isolates can survive for long time in the environment like ventilation systems, humidifier chambers, nebulizers and related devices and accumulate then cause high contamination (Schultsz et al. 2003), therefore the equipments which described previously should be cleaned more repeatedly with disinfectants, for controlling on infections, correct sterilization and cleaning or disinfection with high level of the reusable equipments are essential, after device and it's parts have been disinfected by chemicals they need to be washed via water (Al-Tameemi 2018, Jadhav et al. 2013).

Jadhav et al. (2013) illustrated that percentage of isolated pathogens of these equipments reduced after 70% ethanol disinfection from 75% to 15% and from 87% to 12% for fungi and bacteria respectively.

CONCLUSION

The nebulizers applying by many different patients therefore can play a vital role in controlling on infections within a hospital so have the potency to be a major exporter of infections transition among patients, MRSA are associate with these infections detecting with significant numbers and resistant to many antibiotics, phenotypic detection like; chromogenic agars and cefoxitin DDM are highly recommended for rabid detection, cefoxitin DDM can use as alternative technique for PCR, vancomycin has high effective on MRSA and the best drug for treating bacterial resistance infections.

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