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Detection of β-lactamase and antibiotic susceptibility of clinical isolates of *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus (SA) is the pathogen of greatest concern in the clinical worldwide because of its intrinsic virulence and ability to cause the diverse array of life-threatening infections. In the last two decades or so, antimicrobial resistance has evolved into one of the most formidable problems of infectious diseases. Bacteria acquire resistance to β-lactam antibiotics, most commonly due to the production of enzymes called β-lactamase. This enzyme hydrolyzes the amide bond of β-lactam antibiotics to make them ineffective. In this study, screening of clinical isolates of SA showed β-lactamase activity in 11 clinical isolates (SA-1745, SA-2071, SA-2940, SA-3151, SA-4423, SA-4620, SA-4627, SA-4693, SA-4696, SA-10760, Methicillin-resistant *Staphylococcus aureus* (MRSA), and one wild- type strain (SA-96) by iodometric and nitrocefin disk methods. The antibiotic susceptibility profiles of 12 strains of SA were determined for four β-lactam antibiotics viz. penicillin, ampicillin, cefoxitin and oxacillin. All the clinical isolates showed resistant to these antibiotics. Further, minimum inhibitory concentration (MIC) of four β-lactam antibiotics was also determined by micro-broth dilution assay. The MIC of penicillin, ampicillin and oxacillin against wild-type strain of SA was 0.078 μg/ml, whereas the MIC of cefoxitin against wild-type strain was recorded as 1.25 μg/ml. The MIC of these antibiotics against 11 clinical isolates of SA including methicillin-resistant *Staphylococcus aureus* (MRSA) ranged between 1.95 μg/ml to 1000 μg/ml. All clinical isolates were highly resistant to penicillin and ampicillin followed by cefoxitin and oxacillin. The outcomes of the present study can be useful for the screening of other natural bioactive compounds against these virulent strains.

1. Introduction

Staphylococcus aureus (*S. aureus*), a member of *Micrococcaceae* family, is a gram-positive cocci, often found as part of the normal microflora of the human skin. It is the most imperative pathogen causes several diseases such as pneumonia, meningitis, toxic shock syndrome (TSS), sepsis etc. (Pitkala et al., 2007; Devpriya et al., 2013; Bakir and Ali, 2016). It is responsible for a large number of serious, life-threatening chronic infections that are becoming increasingly dangerous due to the prevalence of antibiotic-resistant strains (Bauer et al., 2013). The *S. aureus* produces an enzyme known as β-lactamase. The staphylococcal β-lactamase hydrolyzes the amide bond of beta-lactam antibiotics that help to acquire the resistance against all beta-lactam antibiotics (DeLeo and Chambers, 2009).

In addition, *S. aureus* expresses methicillin hydrolyzing β-lactamase to acquired resistance against methicillin, which is a semisynthetic derivative of penicillin (Stapleton et al., 2002). The methicillin-resistant *S. aureus* (MRSA) strain is highly pathogenic due to its dual resistance to methicillin and other β-lactam antibiotics that results in high infiltration to the hospitals (HA-MRSA) and community settings (CA-MRSA) (DeLeo and Chambers, 2009). Furthermore, HA-MRSA (Hospital-acquired Methicillin-resistant *Staphylococcus aureus*) infections are the factors that held most responsible for mortality and morbidity in the clinical world (Ragbetli et al., 2016). It can be easily spread from one patient to other and can lead to frequent epidemics. The glycopeptides, vancomycin and teicoplanin are commonly used antibiotics to treats MRSA but resistance such as VRSA (Vancomycin-resistance *Staphylococcus aureus*) and VISA (Vancomycin-intermediate *Staphylococcus aureus*) are now developed against them (Fair and Tor, 2014).

Antibiotic resistance has been referred to as *"the silent tsunami facing*

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modern medicine" (Exner et al., 2017). The antimicrobial drugs have changed the treatment of various infectious diseases. Antimicrobial chemotherapy made remarkable advances and changed the fate of mankind with the optimistic view that infectious diseases would be overcome in the future. However, in reality, emerging and re-emerging infectious diseases are becoming more challenging and problematic infections. Infection with drug-resistant microorganisms are one of the formidable problems in the field of medical sciences and it is becoming more difficult to find solutions of such infections (Saga and Yamaguchi, 2009).

In the middle of the 20th century, major advances happened in the development of antibacterial drug and other means of infection control that helped mankind to overcome the infectious diseases (Tenover et al., 2006). With respect to bacterial infections, the situation improved substantially when penicillin was introduced for clinical use in the early 1940's (Tenover et al., 2006; DeLeo and Chambers, 2009). However, as soon as antibacterial drugs deployed for the treatment, bacteria responded by manifesting various forms of antibacterial drug resistance. Since, the use of antibiotics increased, the levels of complexities in the mechanism of drug resistance by pathogens are also increased in a similar manner that resulted in the selection of antibiotic-resistant bacteria (Tenover et al., 2006; Oryasin et al., 2013; Balsalobre et al., 2014).

At present, antibiotic resistance is becoming a worrisome problem for clinicians worldwide in both hospitals and communities. The overuse or misuse of antibiotics emerge the antimicrobial resistance and developed the multidrug resistance (MDR) in bacterium such as methicillin-resistant (MRSA) in *S. aureus*. Due to the MDR, increased treatment failure and challenges become with a high rate of mortality when compared it to infections caused by susceptible organisms (Bartash and Nori, 2017). The β-lactam is the most important antibacterial agents used in the treatment of bacterial infections. It is generally characterized by the presence of four-membered β-lactam ring which interferes by inhibiting the bacterial cell wall synthesis. A major mechanism of the β-lactam antibiotic resistance is due to the production of an enzyme called β-lactamase that hydrolyzes the amide bond of a β-lactam ring. As a result, antibiotics such as penicillin (6-Aminopenicillanic acid, is unable to inhibit bacterial cell wall synthesis (Moellering, 1993).

The β-lactamase is a plasmid-encoded enzyme produced by a wide range of prokaryotes including *S. aureus*, *Escherichia coli* etc. (Bidya and Suman, 2014). Staphylococcal β-lactamase was among the first enzyme that was found to destroy the penicillin and it has the ability to outwit the human immune system and its multiple drugs resistance phenotype, makes it one of the most impolite pathogenic bacteria in the antimicrobial world (Hiramatsu et al., 2014). To overcome the resistance imposed by β-lactamase producing microorganisms, the β-lactamase inhibitors can be combined with the older β-lactam antibiotics to restore its activity (Gupta et al. 2013). This phenomenon has been reported as an effective strategy for overcoming the mechanism of resistance (Vinod et al., 2010).

Plants are known sources of structurally diverse phytomolecules that play a significant role in drug discovery with a novel mechanism of action (Gupta et al., 2013). The synergistic effects of the phytochemicals in association with antimicrobial agents have already been established (Betoni et al., 2006). Thus, use of these phytochemicals in therapeutic treatments can be a great achievement. Moreover, the exploration of combination therapy reduces the risk of MRSA and other pathogenic bacteremia. It is an effective cure to treat a bacterial infection with low side effects (Niska et al., 2012, 2013; Seah et al., 2013; Ashizawa et al., 2016; Xia et al., 2016). This strategy helps to improves treatment efficacy and play an important role to enhance the value of pre-existing antimicrobials. In this manner, research findings elaborate the role and an alternative approach to reducing the pathogenic bacteria load with the use of naturally derived compounds like honey, Ashoka (Jenkins et al., 2011a, 2011b & 2014; Jenkins et al., 2012; Brudzynski & Lannigan, 2012; Cooper & Jenkins, 2012; Gupta et al., 2013; Muller et al., 2013; Liu et al., 2015).

Several methods have been developed to detect β-lactamase production in the bacteria and also for assaying the activity of β-lactamase. In order to identify the β-lactamase inhibitors which can be used in the treatment of resistant strains of *S. aureus*, an attempt has been made in the present study to standardize the assays for the detection and activity measurement of Staphylococcal β-lactamase enzyme.

2. Materials and methods

2.1. Bacterial strains and antimicrobial agents

The reference strain of *Staphylococcus aureus* MTCC-96 (SA-96) (wild-type) was procured from MTCC (Microbial Type Culture Collection), CSIR-Institute of Microbial Technology (Chandigarh, India). Additionally, 11 clinical isolates of *S. aureus* (SA-1745, SA-2071, SA-2940, SA-3151, SA-4423, SA-4620, SA-4627, SA-4693, SA-4696, SA-10760, MRSA) were obtained from the Clinical Microbiology Laboratory, Sanjay Gandhi Post Graduate Institute of Medical Sciences (Lucknow, India) which were maintained in their repository. The numbers mentioned alongside the strains represent the repository accession number. Clinical isolates of the used strains were characterized and maintained following the procedure suggested by Gupta et al. (2012). Furthermore, antibiotics viz. penicillin, oxacillin, ampicillin, cefoxitin and cephalosporin (Sigma-Aldrich, St. Louis, MO, USA) were used as positive control.

2.2. Isolation of single colony

Staphylococcus aureus (Wild-type and clinical), cultures were streaked on Muller Hinton agar plate and incubated at 37 °C, for overnight (16–18 hrs) to obtain well separate colonies.

2.3. Anti-staphylococcal activity

Following the guidelines of CLSI (2009), determination of the minimum inhibitory concentration (MIC) antibacterial activity of the β-lactam antibiotics was carried out through the micro-dilution broth assay using 96 'U' bottom microtiter plates. To rule out any errors during the procedure, experimental observations were performed in triplicate.

2.4. Detection of β-lactamase in different strains of Staphylococcus aureus

Two different methods were used for the detection of b-lactamase – i) Iodometric strip method and ii) Chromogenic cephalosporin test (Nitrocefin disk method).

2.4.1. Iodometric strip method

Iodometric strip method is based on iodine reacting with penicilloic acid rather than penicillin. In this method, the b-lactam antibiotics i.e. penicillin G (10mg/ml) was dissolved in 0.2% starch solution and the mixture was soaked into Whatman No.1 filter paper (pre-sterilized). Additionally, the strips were moisturized with iodine and 2-3 colonies of *S. aureus* strains were smeared. If the colour of the starch iodine complex was disappeared in 5 minutes, the bacteria were identified as beta-lactamase positive (Devi et al. 2002) (Fig. 1).

2.4.2. Chromogenic cephalosporin method (Nitrocefin disk method)

In the chromogenic cephalosporin method, nitrocefin disks (Himedia Laboratories, India) are impregnated with nitrocefin which shows the rapid detection test of β-lactamase positive strains (Kilic et al. 2006). The colour of nitrocefin disk changed from yellow to red during the process due to the hydrolyzation of the amide bond in a β-lactam ring by a β-lactamase enzyme (Fig. 5). In this method, nitrocefin disk

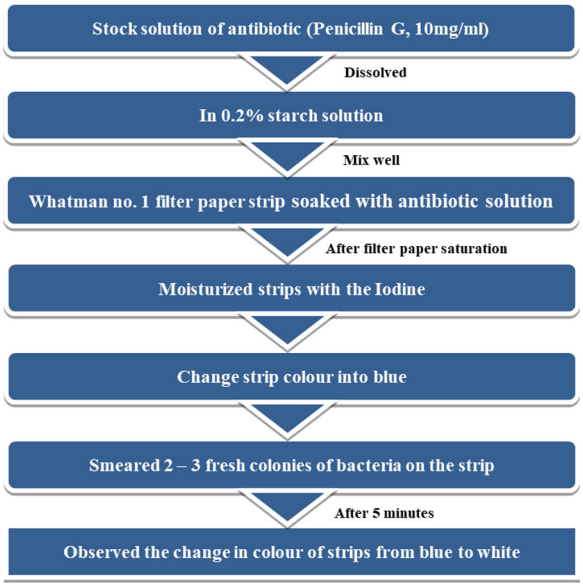

Fig. 1. Successive procedure of chromogenic cephalosporin method for the rapid detection test of β-lactamase positive strains.

was placed on the β-lactamase producing isolates of *S. aureus* and non β-lactamase producing other strain (*Micrococcus luteus*) and incubate at room temperature for 5 minutes. Bacteria which are produced by beta-lactamase in significant amounts, responsible for the change in colour of Nitrocefin Disk (Kilic et al. 2006).

3. Results and discussion

3.1. Antibiotic susceptibility/resistance profiling of clinical isolates of S. aureus

Initially, antibiotics susceptibility of 11 clinical isolates (characterized as methicillin-resistant) and a wild-type strain of *S. aureus* were determined. It is observed that, all the clinical isolates were resistant towards the four members of β-lactam group of antibiotics such as penicillin, ampicillin, cefoxitin and oxacillin (Table 1).

In order to measure the level of resistance, minimum inhibitory concentrations of four β-lactam antibiotics were determined through micro-broth dilution assay.

The Minimum Inhibitory Concentration (MIC) of antibiotics viz. penicillin, ampicillin and oxacillin against wild-type strains of *S. aureus* were observed to be 0.078 μg/ml, whereas the MIC of cefoxitin against wild-type strains was recorded as 1.25 μg/ml. However, the MIC of these antibiotics against 11 clinical isolates of *S. aureus* was found in the range of 1.95 μg/ml to 1000 μg/ml. The clinical isolates observed highly resistant towards penicillin and ampicillin followed by cefoxitin and oxacillin (Table 2).

Table 1
Antibiotic susceptibility/resistance profiles of clinical isolates of *S. aureus* toward β-lactam antibiotics. Symbols represent - PEN for Penicillin, AMP for Ampicillin, CEF for Cefoxitin, OXA for Oxacillin, S for Susceptible and R for Resistant.

Strains	Antibiotics			
	PEN	AMP	CEF	OXA
SA-96	S	S	S	S
SA-1745	R	R	R	R
SA-2071	R	R	R	R
SA-2490	R	R	R	R
SA-3151	R	R	R	R
SA-4423	R	R	R	R
SA-4620	R	R	R	R
SA-4627	R	R	R	R
SA-4693	R	R	R	R
SA-4696	R	R	R	R
SA-10760	R	R	R	R
MRSA	R	R	R	R

3.2. Detection of β-lactamase in various clinical isolates of S. aureus

3.2.1. Iodometric strip method

In this method, the filter paper strip soaked in 0.2% starch (Himedia Laboratories, Mumbai, India) solution containing penicillin was used. When the strips were moisturized with iodine and 2–3 colonies of bacterial strains were smeared on it, change in colour was observed within 5 minutes indicating presence of β-lactamase in the bacterium. All the 11 clinical isolates of *S. aureus* and a wild type strain were observed to produce β-lactamase (Fig. 2).

3.2.2. Chromogenic cephalosporin method

This assay is rapid and can be applied for the detection of β-lactamase enzyme from large number of bacterial strains. This assay was performed by using commercially obtained nitrocefin disk which was observed to change into red colour after inoculation of test bacterium. Change in the colour is indicator for the presence of β-lactamase. Based on the result of this chromogenic cephalosporin method all clinical isolates of *S. aureus* used in this study were found to be β-lactamase producer (Fig. 3). Another bacterium *Micrococcus luteus* used in this study was found to be β-lactamase negative (Fig. 4; Table 3).

The iodometric strip method can be used when chromogenic cephalosporin method is not available. Iodometric test was found to be cheaper, easy to perform and effective in detecting *Staphylococcal* β-lactamase.

3.3. Determining β-lactamase activity using Nitrocefin disk

An attempt was made to purify β-lactamase enzyme from clinical isolate 1745 of *S. aureus*. Since the enzyme could not be purified to homogeneity rich fraction (enzyme solution) was evaluated for β-lactamase activity using nitrocefin disk. It was observed that the colour of nitrocefin disk was changed when it was soaked into 20 μl of enzyme solution within 30 minutes incubation at room temperature. This indicated the presence of β-lactamase enzyme in the solution (Fig. 5).

The β-lactam antibiotics are the most important and frequently used for the treatment of various bacterial infections. Presently, several human pathogenic bacterial strains have acquired resistance towards almost all the antibiotics of this group. Bacteria acquire resistance against β-lactam antibiotics by producing enzyme called as β-lactamase which is capable of degradation of β-lactam antibiotics. According to Bush's classification *Staphylococcal* β-lactamase is classified as group 2a because it hydrolyzes penicillin faster than cephalosporin. β-lactamase acts like serine peptidase which forms an ester bridge with the carbonyl group of β-lactam antibiotic. It breaks the β-lactam ring to form penicilloic acid and an acyl-enzyme complex. The enzyme turns into its initial active structure by simple deacylation which can then bind to other β-lactam antibiotics.

To overcome this issue, identification of β-lactamase inhibitor has become a necessity. β-lactamase inhibitor, when used in combination with the β-lactam antibiotics, which have become non-functional can restore their activity against β-lactamase producing bacteria. β-lactamases are produced by wide range of Prokaryotes. However, Staphylococcal β-lactamase was the first bacterial enzyme identified for degradation of β-lactam antibiotic penicillin.

4. Conclusions

In this study, an attempt has been made to standardize different

Table 2
Minimum Inhibitory Concentration (MIC) values of β-lactam antibiotics against various clinical isolates of *S. aureus* (in μg/ml).

Strains antibiotics	SA-96	SA-1745	SA-2071	SA-2490	SA-3151	SA-4423	SA-4620	SA-4627	SA-4693	SA-4696	SA-10760	MRSA
Penicillin	0.078	1000.000	1000.000	250.000	1000.000	500.000	50.000	1000.000	500.000	250.000	1000.000	31.250
Ampicillin	0.078	1000.000	1000.000	1000.000	1000.000	500.000	500.000	1000.000	250.000	250.000	1000.000	15.150
Cefoxitin	1.250	1000.000	500.000	125.000	500.000	500.000	31.250	250.000	3.900	7.830	500.000	500.000
Oxacillin	0.078	1000.000	1000.000	1000.000	500.000	500.000	31.250	500.000	1.950	1.950	500.000	500.000

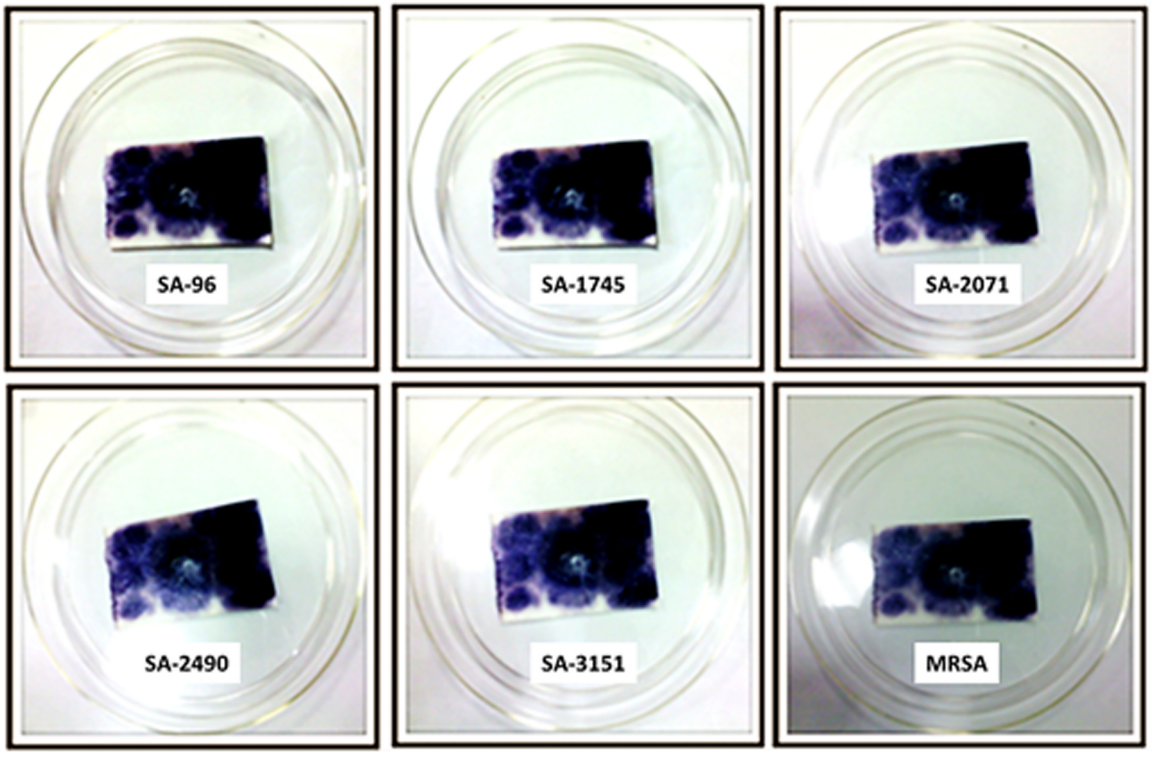

Fig. 2. Detection of β-lactamase in clinical isolates of *S.aureus* using iodometric strip method.

assays for the detection of β-lactamase enzyme from various clinical strains of *S. aureus* to determine the minimum inhibitory concentration of 11 clinical and a wild type isolates of *S. aureus*. Results suggested that all clinical strains are highly resistant against older β-lactam antibiotics in contrast to wild-type. The study will be beneficial for the screening of bioactive compounds isolated from medicinal plants for the evaluation of β-lactamase activity alone or with combination of other β-lactam antibiotics.

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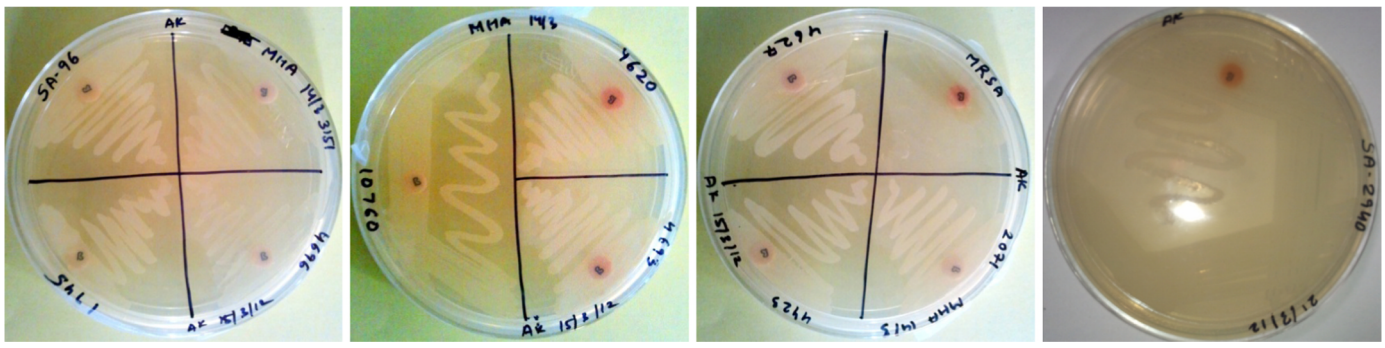

Fig. 3. Detection of β-lactamase in clinical isolates of *S. aureus* using chromogenic cephalosporin method.

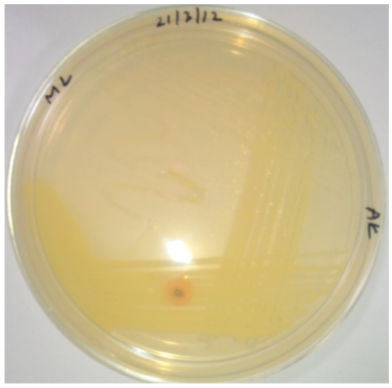

Fig. 4. Absence of β-lactamase in bacterium *Micrococcus luteus*.

Table 3
β-lactamase positive and negative bacterial strains.

S. no.	Strains/isolates	β-lactamase
1.	*Staphylococcus aureus*-96	+ ve
2.	*Staphylococcus aureus*-2940	+ ve
3.	*Staphylococcus aureus*-MRSA	+ ve
4.	*Staphylococcus aureus*-2071	+ ve
5.	*Staphylococcus aureus*-1745	+ ve
6.	*Staphylococcus aureus*-4627	+ ve
7.	*Staphylococcus aureus*-4620	+ ve
8.	*Staphylococcus aureus*-4696	+ ve
9.	*Staphylococcus aureus*-3151	+ ve
10.	*Staphylococcus aureus*-4693	+ ve
11.	*Staphylococcus aureus*-4423	+ ve
12.	*Staphylococcus aureus*-10760	+ ve
13.	*Micrococcus luteus*-2470	− ve

(Indication of β-lactamase: + = present, − = absent).

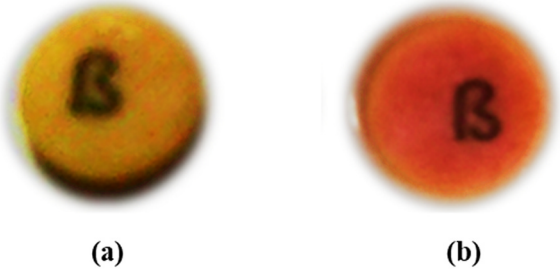

Fig. 5. Determination of β-lactamase activity in enzyme solution using Nitrocefin disk. a) Negative reaction of Nitrocefin disk without enzyme solution and b) Positive reaction of Nitrocefin disk with enzyme solution.

positive environment. AKK acknowledges Prof. K.N. Prasad (Sanjay Gandhi Post Graduate Institute of Medical Sciences) for providing the clinical isolates of methicillin-resistant isolates of *S. aureus*.

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