Methicillin Resistant *Staphylococcus aureus* - Post surgical Infections in Egyptian Hospital

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Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a type of bacteria that is resistant to certain antibiotics including methicillin, oxacillin, penicillin and amoxicillin. Our study investigated the reason of Methicillin Resistant *Staphylococcus aureus* (MRSA) infection in an Egyptian hospital in which multiple drug resistant *S. aureus* was isolated from pus, sputum and blood of infected cases. Our objective was to detect the *mec-A* gene using PCR analysis to confirm that the multiple drug resistant *S. aureus* is MRSA as well to find the drug of choice to be used for competing such infections and to find a safe method for competing MRSA using probiotics. The antibacterial effect of probiotic strains isolated from different animals was tested against MRSA isolates. The results obtained from molecular analysis identified the *mec-A* gene in six out of seven tested samples with a great success with an incidence 85.71%. Moreover, the results revealed that cefobid as well as claforan are the drugs of choice for competing MRSA. *B.subtilus* followed by *L. acidophilus* isolated from colostrum of mare showed great capability of hindrance of MRSA, then *L. palantarum*, *Bifidobacterium* and finally *L.acidophilus* isolated from goat colostrums, *L. acidophilus* isolated from buffalo-cow milk on the contrary showed no activity against MRSA. Our study identified *mec-A* gene from MRSA strains was confirmed to be the main cause of MRSA outbreak in infected hospital patients subjected to stressful conditions due to severe skin infections.
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1.Introduction:

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a type of bacteria that is resistant to certain antibiotics including methicillin, oxacillin, penicillin and amoxicillin (Wichelhaus *et al*. 1997). *S. aureus* remains one of the most intensively investigated bacterial species in human and animal pathogen, it can cause a variety of nosocomial and community-acquired infections ranging from minor skin abscesses to serious potentially life-threatening diseases such as bone and soft tissue intra- surgical infections, sepsis and invasive endocarditis (Chambers, 2001; Enright *et al.* 2002). In terms of resistance, *S. aureus* infections cause an increasing problem. Methicillin-resistant *S. aureus* (MRSA) have spread worldwide, with infection rates >40% in Japan and Southern Europe (Bozdogan *et al.* 2003 and Chang *et al.* 2003) and 20%-25% in the United States (Singh *et al.* 2006).

Conventional methods (culture, biochemical tests and antimicrobial susceptibility) are limited and time consuming. In particular, PCR appears to be a rapid, sensitive, and specific assay for *mec-A* gene (Geha *et al.* 1994). Murakami *et al.* (1991) determined the resistance to methicillin by the *mec-A* gene, which allows a bacterium to be resistant to antibiotics such as methicillin, and other penicillin-like antibiotic, because it does not allow the ring like structure of penicillin-like antibiotics to attack the enzymes that help forming the cell wall of the bacterium, and hence the bacteria is allowed to replicate normally.

Alternative medicine treatments have been practiced for thousands of years around the world, because the effectiveness of currently available antibiotics is decreased due to increasing the number of resistant strains causing infections including MRSA. Lactic acid bacteria (LAB) are famous as friendly bacteria for human health due to their production of bacteriocins (Settanni and Corsetti, 2008).

The aim of the present work is isolation and biochemical characterization of MRSA as well as detection of *mec-A gene* using PCR. MRSA sensitivity was checked against different antibiotics as well as different probiotic strains isolated from different sources.

2. Materials and Methods:

Human clinical sampling:

An investigation was carried out in a hospital showing spread of multiple drug resistant *S. aureus* (18 strains) isolated from different samples including 13 pus (10 males and 3 females) as well as 4 sputum (3 males and 1 female) and 1 blood sample from male as shown in table (1).

Bacteriological sampling and monitoring bacterial profile:

Bacterial swabs were collected under aseptic conditions, cultivation of samples, isolation and purification of the isolates were carried out using different media which were purchased from (Oxoid); swabs were inoculated into a tube containing 10 ml tryptic soy broth. The broth was incubated at 37ºC for 24 hrs then streaked from the enriched broth onto nutrient, mannitol, blood and MacConkey agar plates. Identification of isolates includes morphological examination by Gram's stain (Cruickshank *et al.* 1975). Biochemical identification was carried out according to Collee *et al.* (1996); CDC (2005) including catalase, oxidase, coagulase, gelatin liquefaction, acetone production and sugar fermentation test including; glucose, maltose, lactose, sucrose, sucrose and mannitol with the production of acid without gas. Mannitol fermentation may be used for provisional identification of human *S.aureus*.

***S.aureus* identification and characterization**:

Staphylococcus isolates were streaked onto mannitol salt agar with 2 µg/ml oxacillin and incubated aerobically at 35°C for 48 hrs. Colonies identified as *S. aureus* were diagnosed according to Bottone *et al.* (1984); CDC, (2005). Confirmation of strains was carried out using Staphytect plus dry spot (Oxoid) as latex identification for *S. aureus*.

Antibiotic sensitivity test:

In vitro sensitivity of *S.aureus* strains (18) were done against 15 different Antibiotics was carried out using Agar diffusion antibiotic sensitivity test was carried out for all isolated strains during the outbreak according to Beaney *et al.,* 1970. Interpretation was carried out according to NCCLS, (2002), Antibiotic discs were obtained from Oxoid including B-lactams [penicillin-G (10 units), amoxicillin/clavulinic acid (20/10 µg/ml), cefotaxime (30 µg/ml)] , macrolides [erythromycin (15 µg/ml)], aminoglcosides [gentamicin (10 µg/ml)], fluoroquinolones [ciprofloxacin (5 µg/ml), ofloxacin (5 µg/ml)] cefadroxil (30 µg/ml), cefobid (75mcg), tetracycline (30 µg/ml), tobramycin (10 µg/ml), sulpha/ trimetho (23.75+1.25 µg/ml), amikacin (30 µg/ml) , amoxy/fluclox (25 µg/ml) and claforan (30mcg).The percentages of sensitive, intermediate and resistant are shown in Tables (2).

Polymerase Chain Reaction

DNA extraction from culture samples

DNA from cultured bacteria was extracted Biospin Bacteria Genomic DNA Extraction Kit, Bioflux. Seven multiple drug resistant *S. aureus* were selected for investigating the presence of *mec-A* gene to prove whether the tested strains are MRSA or not. Single colonies of isolates were cultured in Luria-Bertani medium and incubated for 16 h at 37ºC. An aliquot (4 ml) of overnight culture (10^9 CFU) was pelleted by centrifugation (13.000 rpm for 4 min). Bacterial pellet was resuspended in 100µl of Elution buffer and extraction was carried out according to instruction by Bioflux Company using (Biospin Bacteria Genomic DNA Extraction Kit).

PCR amplification:

On the basis of the DNA sequences of the *mec-A* gene, the following oligonucleotides were used in PCR amplification: primers M1 (TGG CTA TCG TGT CAC AAT CG) and M2 (CTG GAA CTT GTT GAG CAG AG), which amplified a 310-bp fragment of the *mec-A* gene (Vannuffel *et al.* 1995). The amplification was carried out using Pyrostart Fast PCR Master Mix (Fermentas Company). Molecular and conventional tests were performed in different laboratories and the results were compared.

***In vitro* antimicrobial activity of probiotic bacteria against MRSA using well diffusion assay (Sgouras *et al.*, 2004):**

mec-A gene positive MRSA strains were tested for their sensitivity toward different probiotic strains as follow; Bifidobacterium, *L.palantarum, L. acidophilus* (buffaloe –cow milk, cow milk, mare colostrum as well as goat colostrum), *B. suibtlus*. Strains were plated onto Mueller Hinton agar plates. The *In vitro* antibacterial activity of the tested probiotic strains using agar well diffusion test was carried out as follow; Muller Hinton agar plates were prepared and wells were drilled out using Pasture pipettes, the plates were inoculated with MRSA strains prepared in conc. equivalent with 0.5 MacFerland tube and streaked onto the agar plates using sterile swabs, and then 50 µl aliquots of cell free cultures supernatant in fresh DeMan Rogaso Sharpe (M.R.S.) broth of the probiotic strains were suspended in the agar wells. Plates were incubated at 37˚C for 24hrs under aerobic conditions and the diameters of inhibition zones around wells were measured in mm using a ruler. The experiment was carried out in duplicate and the mean of the zone of inhibition was estimated as follows; ++ showed zone of inhibition ≥ 8 mm, + showed zone ≤ 7mm and – indicate complete absence of inhibition.

3. Results

Traditional analysis of 18 samples collected from post-surgical side infected patients including; 14 males and 4 females (13 pus, 1 blood and 4 sputum) Table (2) were carried out using bacterial isolation and biochemical identification. Results revealed the presence of Gram positive, non-spore forming cocci, arranged in form of grapes or in irregular clusters. The colonies are circular, smooth and glistening. On blood agar, they are beta-hemolytic. Colonies are golden yellow. Biochemically; they were catalase, coagulase positive and mannitol fermenter which proved to be *S. aureus*. Antibiotic sensitivity test was

carried out using fifteen antibiotic disks (Oxoid). *In vitro* antibiotic sensitivity test against eighteen *S. aureus* isolated strains, showed resistance against cefotaxime, cefadroxil, sulph/trimetho and amoxy/fluclox with an incidence equal 94.40%. Resistance against pencillin–G, erythromycin, gentamycin, tetracycline and amickacin was shown with an incidence 89.00% and resistance aganist amoxicillin/clavulinic acid, ciprofloxacin and ofloxacin with an incidence 83.30%. Drugs that were capable of hindrance of such strains were cefobid and claforan with an incidence of 83.30%, thus they were used successfully for treatment of infected cases as shown in Table (2, 3). Molecular analysis was carried out for detection of *mec-A* gene which was responsible for multiple drug resistance of the tested strains to confirm that tested strains were MRSA. Results revealed that the isolated strains from pus samples of patients subjected to stressful conditions due to post operative infections were MRSA; Molecular analysis identified the *mec-A* gene in six out of seven samples with an incidence equal 85.71% as shown in Photo (1). On investigating the use of probiotics for competing MRSA as a safe method for hindrance of multiple drug resistant strains, results revealed that *B.subtilus* isolated from mare fecal swab followed by *L. acidophilus* isolated from mare colostrum showed great capability of hindrance of MRSA, followed by *L. palantarum* then *Bifidobacterium* and finally *L.acidophilus* isolated from goat colostrums. On the contrary, *L. acidophilus* isolated from buffalo-cow milk showed no activity against MRSA as shown in Photos (2, 3) and Table (4).

Table (1): Diagnosis of Cases showing multiple drug resistant *S. aureus* and site of sample collection.

Sample type	Case Diagnosis	Male	Female	Total
Pus	**Burn**	0	1	1
	Boil	2	1	3
	SSI	4	1	5
	Bone Fracture	2	0	2
	Plastic flap	1	0	1
	Osteomyelitis	1	0	1
Sputum	**Cancer Larynx**	1	0	1
	Chest infection	2	0	2
	Stroke	0	1	1
Blood	**Head trauma**	1	0	1
	Total	14	4	18

Table (2): Antibiotic sensitivity test against *S. aureus* (18 strains)

Antimicrobial agent	Conc. of disk	Sensitive		Intermediate		Resistant	
		No	%	No	%	No	%
B-lactams							
Penicillin-G	10 units	0	0.00	2	11.00	16	89.00
Amoxicillin/clavulinic acid	20/10 µg/ml	0	0.00	3	16.70	15	83.30
Cefotaxime	30 µg/ml	0	0.00	1	5.60	17	94.40
Macrolides							
Erythromycin	15 µg/ml	0	0.00	2	11.00	16	89.00
Aminoglcosides							
Gentamicin	10 µg/ml	0	0.00	2	11.00	16	89.00
Fluoroquinolones							
Ciprofloxacin	5 µg/ml	0	0.00	3	16.70	15	83.30
Ofloxacin	5 µg/ml	0	0.00	3	16.70	15	83.30
Cefadroxil	30 µg/ml	0	0.00	1	5.60	17	94.40
Cefobid	75mcg	15	83.30	3	16.70	0	0.00
Tetracycline	30 µg/ml	0	0.00	2	11.00	16	89.00
Tobramycin	10 µg/ml	0	0.00	4	22.00	14	78.00
Sulpha/ trimetho	23.75+1.25 µg/ml	0	0.00	1	5.60	17	94.40
Amikacin	30 µg/ml	0	0.00	2	11.00	16	89.00
Amoxy/fluclox	25 µg/ml	0	0.00	1	5.60	17	94.40
Claforan	30mcg	15	83.30	3	16.70	0	0.00

Table (3): *S. aureus* strains isolated from pus samples tested for *mec-A* gene presence and antibiotics used for cases treatment.

PCR (pus) Sample	Age (years)	Sex	Diagnosis	Antibiotic used for treatment
1	30	male	Bone fracture	Claforan and Cefobid
2	8	male	Bone fracture	Claforan and Cefobid
3	30	male	Plastic flap	Claforan and Amikane
4	44	male	SSI	Claforan and Cefobid
5	22	male	SSI	Claforan and Cefobid
6	10	male	SSI	Claforan and Cefobid
7	41	female	SSI	Claforan and Cefobid

SSI=Surgical side infection

Table (4): The antimicrobial activity of the tested probiotic strains against MRSA.

Probiotic strains	Source	Tested MRSA strains						
		1	**2**	**3**	**4**	**5**	**6**	**7**
B.subtilus	fecal swab of mare	++	++	+	+	+	++	++
L. acidophilus	buffaloe-cow milk	-	-	-	-	-	-	-
Bifidobacterium	cow milk	+	+	+	-	-	+	+
L. acidophilus	goat colostrums	+	-	-	-	-	-	-
L. acidophilus	mare colostrum	++	++	+	+	+	+	+
L. palantarum	cow milk	++	++	+	-	+	+	+

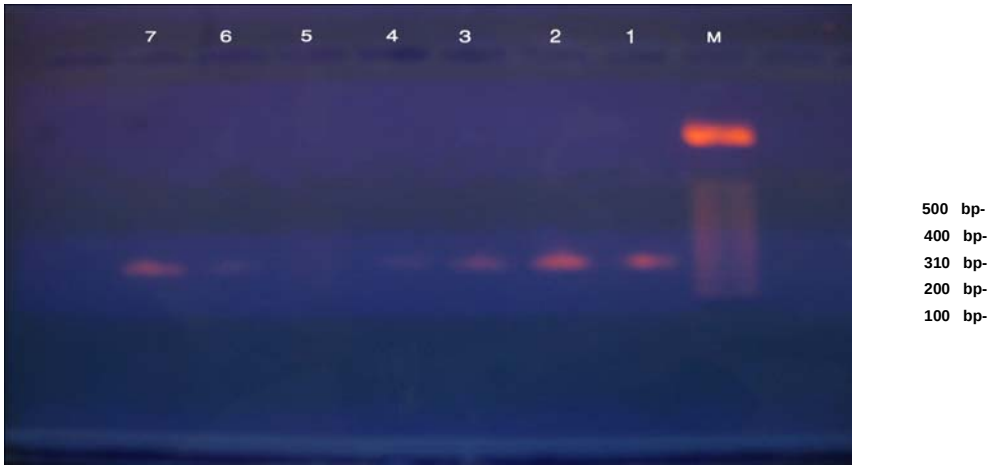

Photo (1) Gel electrophoresis of DNA fragments showing 310 bp amplified fragment of *mec-A* gene among the examined *S.aureus* isolated from hospitalized patients. Lane M represents DNA ladder. Lanes 1 to 7 represent *S. aureus* isolated strains as follow; (1) Pus from bone fracture of male, (2) Pus from bone fracture of male, (3) Pus from plastic flap of male, (4) Pus from surgical side infection of male, (5) Pus from surgical side infection of male (6) Pus from surgical side infection of male, (7) Pus from surgical side infection of female

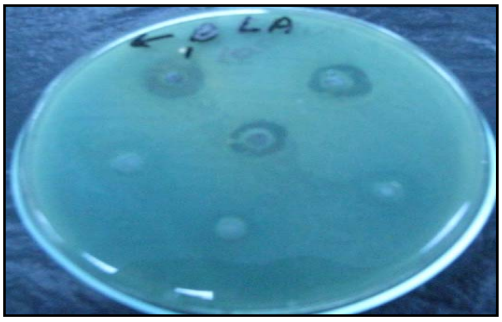

Photo (2) MRSA strain (1) isolated from pus of human, show variable sensitivity toward *B.subtilus* isolated from the fecal swab of mare, followed by *L. acidophilus* isolated from colostrum of goat, *Bifidobacterium* isolated from cow milk sample, *L. acidophilus* isolated from Buffalo-cow milk then *L. acidophilus* isolated from colostrum of mare, *L. palantarum* isolated from cow milk, in sequence with the arrow.

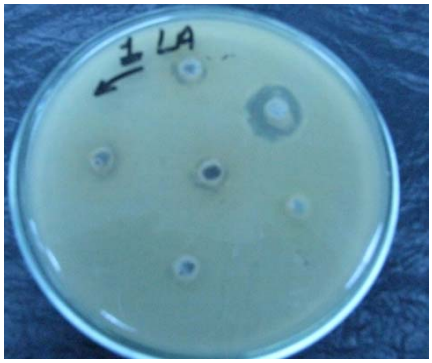

Photo (3) MRSA strain (2) isolated from pus of human, show variable sensitivity toward *B.subtilus* isolated from the fecal swab of mare, followed by *L. acidophilus* isolated from colostrum of goat, *Bifidobacterium* isolated from cow milk sample, *L. acidophilus* isolated from Buffalo-cow milk then *L. acidophilus* isolated from colostrum of mare, *L. palantarum* isolated from cow milk, in sequence with the arrow**.**

4. Discussion

The present study was carried out to investigate the cause of development of multiple drugs resistant strains as side infection following surgical operations in an Egyptian hospital. Results showed that the outbreak was due to infection with multiple drug resistant *S. aureus* (MRSA). This study proved that MRSA is an opportunistic pathogen which is abused of being a critical pathogen responsible for a great morbidity especially among immunosuppressed cases. MRSA outbreaks in hospitals suggested that this organism cause an emerging problem in patients subjected to multiple stressful conditions. Results agree with Hudson (1994) and Cookson (1998) who proved that the treatment of *S. aureus* infections may be complicated by multiple antibiotic resistances and specific virulence factors, causing temporary or long-lasting carriage. The nasal carriage of MRSA is a main risk factor for community-acquired infections and in hospital settings (nosocomial sepsis). Also, results agree with Hiramatsu *et al.* (2002) who found that infection rate in carriers of *S. aureus* is higher than in non-carriers, and it has been well documented that humans are usually infected with their own nasal isolate. Results also agree with Gomez-Lucía *et al.* (1989); Kloos and Bannerman (1999) who mentioned that *S. aureus* is an opportunistic pathogen which can cause diseases ranging from superficial soft-tissue infections to life-threatening bacteraemia and toxic shock syndrome. These findings agree with Quinn *et al.* (2002) who abuse MRSA of being a critical pathogen responsible for a great morbidity and mortality especially among immunosuppressed cases.

Antibiotic sensitivity test against eighteen isolated strains showed resistant against cefotaxime, cefadroxil, sulph/trimetho and amoxy/fluclox with an incidence equal 94.40%. Resistance against pencillin –G, erythromycin, gentamycin, tetracycline and amickacin was shown with an incidence 89.00%. Amoxicillin/clavulinic acid, ciprofloxacin and ofloxacin were resistant with an incidence 83.30%, on the other hand, the tested strains were sensitive against cefobid and claforan with an incidence of 83.30%. These results agree with Quinn *et al.* (2002) who proved that MRSA either produce potent toxins or resist a wide range of antibiotics. Results agree with Karska-Wysocki *et al.* (2010) who showed that MRSA is a multidrug-resistant microorganism and the principal nosocomial pathogen worldwide. Tiwari *et al.* (2009) compared the performances of four phenotypic tests used to detect methicillin resistant *S. aureus* (MRSA) with the *mec-A* gene polymerase chain reaction. Two hundred thirty-seven *S. aureus* isolates were isolated from different patients visiting Hospital and subjected to cefoxitin and oxacillin disc diffusion tests, oxacillin minimum inhibitory concentration (MIC) test, and oxacillin screen agar test. The authers stated that cefoxitin disc diffusion test can be considered as the best method for routine detection of MRSA when molecular techniques are not available.

Molecular analysis performed in the present study was necessary to assess the feasibility of the PCR approach for the identification of *S. aureus* multiple drug resistant strain. Our study revealed that PCR analysis verified that 85.71% of the tested strains carried *mec-A* gene at 310 bp fragment. On the basis of these results, the PCR strategy could give rapid and reliable information to clinicians not only for the identification of pathogenic bacteria but also for therapeutic management. Results agree with Montanari *et al.* (1990) who explained the absence of

mec-A gene in the 25 MRSA isolates by overproduction of penicillinase. Also, Oshima *et al.* (1993) amplified *mec- A* gene by PCR and reported that the gene was positive in all MRSA strains. They reported that identification of MRSA by drug susceptibility tests alone presented a serious problem because numbers of clinical *S. aureus* isolates are border line resistant to methicillin. Hence quick, accurate and sensitive method of PCR based amplification for the detection of the *mec-A* gene is necessary. They added that detection of *mec-A* gene by PCR is extremely important for appropriate treatment of MRSA. Our results agree with Vannuffel *et al.* (1995) who indicated that MRSA has become a major nosocomial pathogen not only in tertiary care hospitals but also in chronic care facilities. Also results agree with Anderson and Weese (2006) who found that conventional identification of MRSA requires between 24-48 hours after sampling and recommended rapid and sensitive method of identification as PCR for detection of *mec-A* gene which codes for the drug resistant penicillin-binding protein 2a(PBP2a) or 2(PBP2). Klotz *et al.* (2005) reported an increase in the frequency of MRSA as an important causative agent of nosocomial infections worldwide, in spite of optimal hygienic conditions high number (24.1%) was isolated from human stool samples.

The present study focused on studying the effect of different probiotic strains and tested their efficiency on hindrance of the growth of MRSA, Results revealed that *B. subtilus* isolated from fecal swab of mare followed by *L. acidophilus* isolated from colostrum of mare showed great capability of hindrance of MRSA, then *L. palantarum*, *Bifidobacterium* isolated from milk and finally *L.acidophilus* isolated from goat colostrum. On the contrary *L. acidophilus* isolated from Buffalo-cow milk showed no activity against MRSA. Results agree with Karska-Wysocki *et al.* (2010) who tested the antibacterial activity of lactic acid bacteria against MRSA from ten human clinical isolate using *L. acidophilus* CL1285® and *L. casei* LBC80R as pure cultures. They demonstrate that the direct interaction of lactic acid bacteria and MRSA in a mixture led to the elimination of 99% of the MRSA cells after 24 h of their incubation at 37 °C.

5. Conclusion and Recommendations

Misuse of antibiotics must be controlled as it might be the main cause of outbreaks due to their immunosuppressive effect on infected cases. Rapid diagnosis should be carried accurately including screening of unusual causes of multiple drug resistant strains in hospitalized patients. Researchers recommended that hospitals should initiate surveillance programs for MRSA infections using quantitative PCR, particularly in post surgical operations to clarify the role of MRSA in drug resistant outbreaks. Research group advice the use of probiotics as *B.suibtlus* or *L.acidophilus* as feed additives in human subjected to stressful conditions for hindrance of opportunistic microorganisms during stressful conditions.

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