Rapid identification of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Directly from Nasal Swab Specimens using duplex Light cycler PCR in Sohag University Hospital

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Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) has spread worldwide and is responsible for significant morbidity, mortality, and health care costs. Control strategies to limit the emergence and spread of this organism rely on rapid and sensitive tests for detection of MRSA carriage. Screening for colonization with methicillin-resistant *Staphylococcus aureus* (MRSA**)** is a key aspect of infection control to limit the nosocomial spread of this organism. Current methods for the detection of MRSA in clinical microbiology laboratories, including molecular methods for the identification of MRSA which are based on the detection of *S. aureus*-specific gene targets and the *mecA* gene require a culture step and the isolation of pure colonies that result in a minimum of 48 to 72 hours until a result is known. Also, clinical specimens such as nasal or wound samples are often colonized by both *S. aureus* and coagulase-negative staphylococci (CoNS) which can both carry the *mecA* gene. In this study, we used two methods for identification of nasal carriage of MRSA; conventional culture methods and duplex real time PCR among one hundred newly admitted patients to Sohag University Hospital (65 males and 35 females), with age range from (25-35 years) in the period from September 2011 to February 2012. The isolates were identified as *Staphylococcus aureus* based on morphology, Gram staining, coagulase and catalase tests, and mannitol salt agar fermentation. Confirmed *S. aureus* isolates were subjected to cefoxitin disc diffusion test using a 30 µg disc to be identified as MRSA. In total, 26 patients (26%) were found to be nasal carriers of *S. aureus* and all these strains were MRSA. These conventional methods took 24-48 hours for routine culture and then further 24 hours for susceptibility testing for identification of MRSA. By using a duplex real-time fluorescence-based PCR assay targeting the *mecA* gene and the *Sa442* gene which allows a rapid detection of nasal colonization with MRSA directly from nasal swab specimens containing a mixture of staphylococci from one hundred newly admitted patients; with the time from the start of processing of specimen to result was approximately three hours. 26 patients (26%) were found to be nasal carriers of MRSA. Duplex real-time fluorescence-based PCR assay is as effective test as the conventional methods; in addition it is more rapid, sensitive and specific for detection of nasal colonization with MRSA and providing for same-day results, allowing more efficient and effective use of infection control resources to control MRSA spread in health care facilities.

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Key words: MRSA, Nasal swab, duplex Light cycler PCR.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important nosocomial pathogens worldwide, and it causes significant morbidity and mortality ***(Aires et al., 2003).*** *Staphylococcus aureus* represents the most common bacterial pathogen isolated from blood cultures and is an important cause of endocarditis, osteomyelitis, pneumonia, and skin and soft tissue infections ***(Lowy, 1998)***. When compared with bacteremias due to methicillin-susceptible *S. aureus* (MSSA), MRSA bacteremias resulted in a 3-fold increase in relative cost and a 3-fold excess length of hospital stay ***(Abramson et al., 1999)***. Once colonized with MRSA, 11 to 25% of patients in acute-care facilities and 3 to 15% in chronic-care facilities subsequently will develop infection ***(Marschall and Mühlemann, 2006)***. Nasal carriage of *S. aureus* has been suggested as the source of bacteremia, surgical-site, and other infections and a reservoir of *S. aureus* in hospitals ***(von Eiff et al., 2001)***. Early detection followed by decolonization with topical mupirocin may prevent infections and reduce transmission ***(Hudson, 1994)***. Screening patients for *S. aureus* colonization using culture methods requires 1 to 4 or more days for accurate detection and identification of *S. aureus* ***(Hacek et al., 2003).*** Over the past several years, a variety of DNA-based tests have been developed to detect MRSA carriage more quickly ***(Levi et al., 2003)***; which is essential for timely decisions on isolation procedures and effective antimicrobial chemotherapy. Most of these assays are based on the detection of an *S. aureus*-specific gene and the *mecA* gene, which encodes methicillin (and oxacillin) resistance. However, none of these molecular tests are suitable for detection of MRSA directly in specimens obtained during nasal screening, because such specimens often contain a mixed flora of coagulase-negative staphylococci and *S. aureus*, both of which can carry *mecA* ***(Fang, and Hedin, 2003)***. By targeting MRSA-specific chromosomal sequences, we were able to develop a real-time PCR assay for the detection of MRSA that allowed us to discriminate MRSA from

methicillin-resistant coagulase-negative staphylococci ***(Huletsky et al., 2004)***. The results of a real-time polymerase chain reaction assay to detect MRSA could be obtained within 3 hours. This unique and rapid PCR test is sufficiently sensitive and specific for direct detection of MRSA in nasal swab specimens. The purpose of this study was to detect MRSA colonization directly in nasal swab specimens collected from newly admitted patients at Sohag University Hospital followed by decolonization with topical mupirocin to prevent infections and reduce transmission.

2. Material and Methods

This study was carried out in Sohag University Hospital during the period from September 2011 to February 2012. One hundred nasal swabs were collected from patients newly admitted to Sohag University Hospital over the study period; (65 males and 35 females), with age range from (25 to 65 years). Patients were excluded from the study if they had received treatment with intranasal mupirocin in the previous 14 days, treatment with oral antimicrobials within the past 14 days (including oral rifampin® therapy for any reason), or had contraindications to nasal sampling. Two sets of sterile cotton swabs were rubbed over the anterior nares of both nostrils of all studied patients; one swab for culture and the other swab for PCR assay; both nostrils were sampled using the same swab. The swabs were immediately sent to the laboratory. One collection swab was initially inoculated into *S. aureus* selective agar and the second into a buffer solution for the MRSA DNA extraction.

Selective media and culture conditions:

Mannitol salt agar plates (bioMerieux, France) were inoculated directly with specimen swabs. The mannitol salt agar plates were incubated for 24 to 48 hrs at 37°C and examined for growth. Strains that produced yellow colonies on mannitol salt agar were confirmed as *S. aureus* with Gram staining, 3% catalase testing, and slide and tube coagulase testing.

Detection of MRSA by culture methods:

Confirmed *S. aureus* isolates were subjected to cefoxitin disc diffusion test using a 30 µg disc. A 0.5 Mc Farland standard suspension of the isolate was made and lawn culture done on Mueller-Hinton agar plate. Plates were incubated at 37 ^{0} C for 18 to 24 hrs and zone diameters were measured. An inhibition zone diameter of ≤ 19 mm was reported as oxacillin resistant and ≥20 mm was considered as oxacillin sensitive. (Based on the recommendations of Clinical Laboratory Standards Institute) ***(CLSI, 2011).***

Detection of MRSA by Real-time PCR:

a) Sample Treatment & DNA Extraction:

DNA was extracted according to the manufacturer's associated instructions by the use of: QIAamp DNA Mini Kit (250) Catalog No.51306 (Qiagen GmbH, Hilden, Germany). The commercially available kit contained the following solutions in a ready-to-use formulation: buffer ATL, buffer AL, buffer AW1, buffer AW2, buffer AE, and proteinase K (20mg/ml).

1. In a 2 ml microcentrifuge tube, 360 µl of Buffer ATL was added.
2. 40µl Proteinase K was added; it was mixed by vortexing.
3. Swab was put & rotated in the tube carefully & after multiple rotations the swab was pressed carefully against the wall of the tube.
4. The solution was mixed by vortexing and incubated at 56°c for 15 min.
5. Then the samples were boiled for 3min in boiling water bath and subjected to freezing for 1min, and repeat this procedure (boiling & freezing) 5 times.
6. After reaching the room temperature, 400µl Buffer AL was added to the sample**.**
7. It was mixed by pulse-vortexing for15s, and incubated at 70°c for 2 min.
8. The microcentrifuge tube was centrifuged briefly to remove drops from inside the lid. It was essential that the sample and buffer AL were mixed thoroughly to yield a homogeneous solution.
9. 400µl ethanol (96-100%) was added to the sample, and mixed by pulse-vortexing for 15s. After mixing, the microcentrifuge tube was briefly centrifuged to remove drops from inside the lid.
10. The mixture from the last step was applied (including the precipitate) to the QIAamp spin column (in a 2ml collection tube) without wetting the rim. The cap was closed, and was centrifuged at 8000 rpm for 1min. The QIAamp spin column was placed in a clean 2ml collection tube and the tube containing the filtrate was discarded.
11. The QIAamp spin column was opened and 500µl Buffer AW1 was added without wetting the rim (Buffer AW1, wash buffer1, contains guanidine hydrochloride, 95 ml concentrate, before use 125ml ethanol was added to obtain 220ml buffer AW1, Lot No.1154869, Mat.No.1014790, QIAGEN GmbH), the cap was closed and was centrifuged at 8000 rpm for 1min. The QIAamp spin column was placed in a clean 2ml collection tube and the collection tube containing the filtrate was discarded.
12. The QIAamp spin column was opened and 500µl Buffer AW2 was added without wetting the rim(Buffer AW2, wash buffer2, 66ml concentrate, before use 160ml ethanol was added to obtain 226ml buffer AW2, Lot No.11243725, Mat.No. 1014592, QIAGEN GmbH), the cap was closed and was centrifuged at full speed; 14000 rpm for 3min.

13. The QIAamp spin column was placed in a clean 1.5ml microcentrifuge tube and the collection tube containing the filtrate was discarded.
14. The QIAamp spin column was opened and 150µl of Buffer AE was added (Buffer AE, Elution buffer, Lot No.11244983, Mat.No.1014574, QIAGEN GmbH), and was incubated at room temperature for 1min, and then was centrifuged at 8000rpm for 1min.
15. The column was discarded and now the 1.5ml microcentrifuge tube containing the isolated DNA.

b) Master Mix preparation:

Kit contents: LightCycler- FastStart DNA Master Mix: Contains FastStart Taq DNA polymerase reaction buffer, dNTP mix {dATP, dCTP, dGTP, dTTP}. Sterile water: Each vial contains 1ml sterile water, PCR grade to adjust final reaction volume. And MgCl2 solution (1x1ml; 25mM MgCl2 solution). Catalog No. 3 335 038 (Roche Diagnostics GmbH, Germany).

Equipment used:

LightCycler Instrument (Roche). (Cat. No. 2 011 468), LightCycler capillaries (cat. No. 1 909 339), and LC Carousel Centrifuge (cat. No. 1 909 282).

The LightCycler system has been designed to reduce the time taken to achieve results from PCR reactions and to enable the user to monitor the amplification of the PCR product simultaneously, in real-time.

Duplex Real-time PCR: (*Reischl et al., 2000)* and *(Grisold et al., 2002*).

The real-time PCR assay was carried out with the LightCycler system (Roche). Primers Sa442-F and Sa442-R, directed to the *Sa442* gene, a *S. aureus*-specific marker and primers MecA-F and MecA-R targeting the *mecA* gene, were used ***(Reischl et al., 2000)***. Oligonucleotide primers and fluorescence-labeled hybridization probes, designed for amplification and sequence-specific detection of a 188-bp fragment within the *mecA* gene and a 178-bp fragment within an *S. aureus*-specific genomic marker ***(Grisold et al., 2002)***, were obtained from Metabion GmbH, Munich, Germany. Nucleotide sequences and positions are listed in **Table 1**. Amplification mixtures contained 2 ul of 10x LightCycler FastStart DNA Master Hybridization Probes mixture (Roche), 3.2 µl $MgCl_2$, 0.1 µl each Mec-F and Mec-R primer oligonucleotide, 0.05 µl each Sa442-F and Sa442-R primer oligonucleotide, 1.33 µl each hybridization probe oligonucleotide, and 5 ul of template DNA in a final volume of 20 ul. The duplex approach, containing four different primer oligonucleotides and four different hybridization probes within a single capillary. The LightCycler capillaries were sealed. Then, the sample carousel with the capillaries was centrifuged in the LightCycler carousel centrifuge and placed into the LightCycler instrument. The cycling protocol consisted of an initial denaturation at 95°C for 10 min to activate the FastStart *Taq* DNA polymerase, followed by 50 cycles consisting of denaturation for 10 s at 95°C, annealing for 10 s at 50°C, and elongation for 20 s at 72°C. After the final cycle, the capillaries were cooled for 2 min at 40°C ***(Grisold et al., 2002)***. Fluorescence curves were analyzed with the LightCycler software (version 3.5.3). The fluorescence of each capillary was measured at wavelengths of 640 and 705 nm (dual-color option). Each result was confirmed by the specific peak in the corresponding melting curve.

Table 1: Oligonucleotide primers and LightCycler hybridization probes used in the PCR assay. *(Grisold et al., 2002)*

Oligonucleotide	Sequence[a]	Target gene	Nucleotide positions	source
Sa442-F	GTCGGGTACACGATATTCTTCACG	*Sa442*	12-34	*(Reischl et al., 2000)*
Sa442-R	CTCGTATGACCAGCTTCGGT	*Sa442*	189–168	*(Grisold et al., 2002)*
Sa442-HP-1	TACTGAAATCTCATTACGTTGCATCGGAA-FAM	*Sa442*	95-123	*(Reischl et al., 2000)*
Sa442-HP-2	Red 705-ATTGTGTTCTGTATGTAAAAGCCGTCTTG-Ph	*Sa442*	126-154	*(Reischl et al., 2000)*
Mec-F	CTAGGTGTGGTGAAGATATACCA	*mecA*	1596–1619	*(Grisold et al., 2002)*
Mec-R	TGAGGTGCGTTAATATTGCCA	*mecA*	1783–1763	*(Grisold et al., 2002)*
Mec-HP-1	CAGGTTACGGACAAGGTGAAATACTGATT-FAM	*mecA*	1690-1718	*(Reischl et al., 2000)*
Mec-HP-2	Red 640-ACCCAGTACAGATCCTTTCAATCTATAGCG-Ph	*mecA*	1720-1739	*(Reischl et al., 2000)*

[a] FAM, fluorescein; Red 705, LightCycler Red 705 phosphoramidite; Ph, 3'-phosphate; Red 640, LightCycler Red 640 *N*-hydroxysuccinimide ester.

Data analysis:

The identity of the PCR product from a sample can be confirmed by performing a melting curve analysis comparing its melting temperature (T_m) with the T_m of the product from the positive control. In this study melting curve analysis was performed to determine which specific gene(s) had been detected from the samples. Strains with T_ms within the range of the positive control's T_m (*mecA*) 66° C ± 1°C and within the range of the positive control's T_m (*Sa442*) 67°C ± 1°C were regarded as *mecA* and *Sa442* positive, respectively.

3. Results

The present study was carried out in Sohag University Hospital during the period from September 2011 to February 2012. One hundred nasal swabs were

collected from newly admitted patients to different wards of Sohag University Hospital (65 males and 35 females); with age range from (25 to 65 years). Nasal screening identified 26 (26 %) MRSA carriers and 74 (74%) were negative for MRSA nasal colonization. MRSA nasal colonization was more in males 15 (57.7%) than females 11 (42.3%).

Culture results:-

A total of 100 nasal swabs were screened for MRSA with the above mentioned methods. The *Staphylococcus aureus* isolates had given yellow colonies on mannitol salt agar. There were 26 *Staphylococcus aureus* isolates identified through conventional methods of culture on mannitol salt agar and then Gram staining (Gram positive cocci arranged in grape-like clusters), catalase test and coagulase test (both slide & tube) positive. All the isolated *S. aureus* strains were identified as MRSA by cefoxitin disc diffusion test using a 30 µg disc. The conventional methods took 24-48 hours for routine culture and then further 24 hours for susceptibility testing for identification of MRSA

Age and sex distribution among the study group:

Sixty five males and 35 females with age range from 25 to 65 years. Maximum number of MRSA nasal carriers (around 62%) were isolated between the age groups of 35-55 years. According to MRSA positive isolates, MRSA nasal colonization was more in males 15 (57.7%) than females 11 (42.3%).

Table (2): Age distribution among the study group.

Age groups	Number	Percentage %
25 - < 35	5	19.2
35 - < 45	7	27
45- < 55	9	34.6
55- < 65	5	19.2
Total	26	100

Duplex real-time PCR results:-

These results were confirmed by the duplex real-time PCR assay directed to the *Sa442* gene; a *S. aureus*-specific marker and targeting the *mecA* gene; a methicillin resistance marker. The whole molecular assay was completed within 3 h compared to 2 to 3 days required by the culture method with a sensitivity and negative predictive value of 100%. This PCR test allows direct detection of MRSA in clinical specimens containing a mixture of staphylococci, without previous isolation, or enrichment of the bacteria, thereby reducing the number of sample preparation steps and the time required to obtain results.

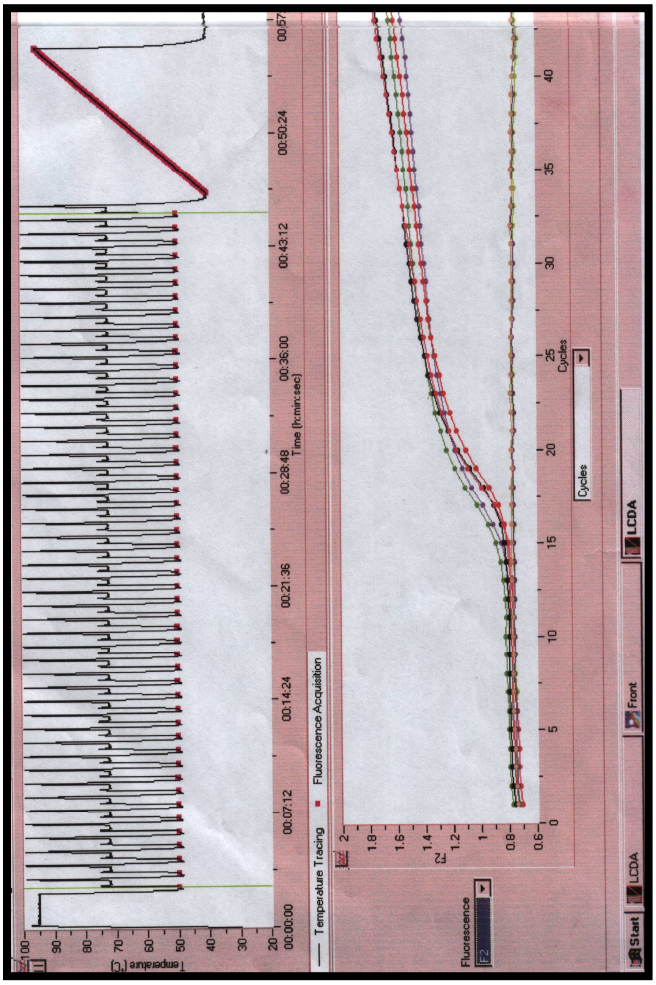

Figure 1: Results of real-time PCR for detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in nasal specimens. The product of the MRSA-specific amplification is measured in terms of increases in fluorescence during the amplification process.

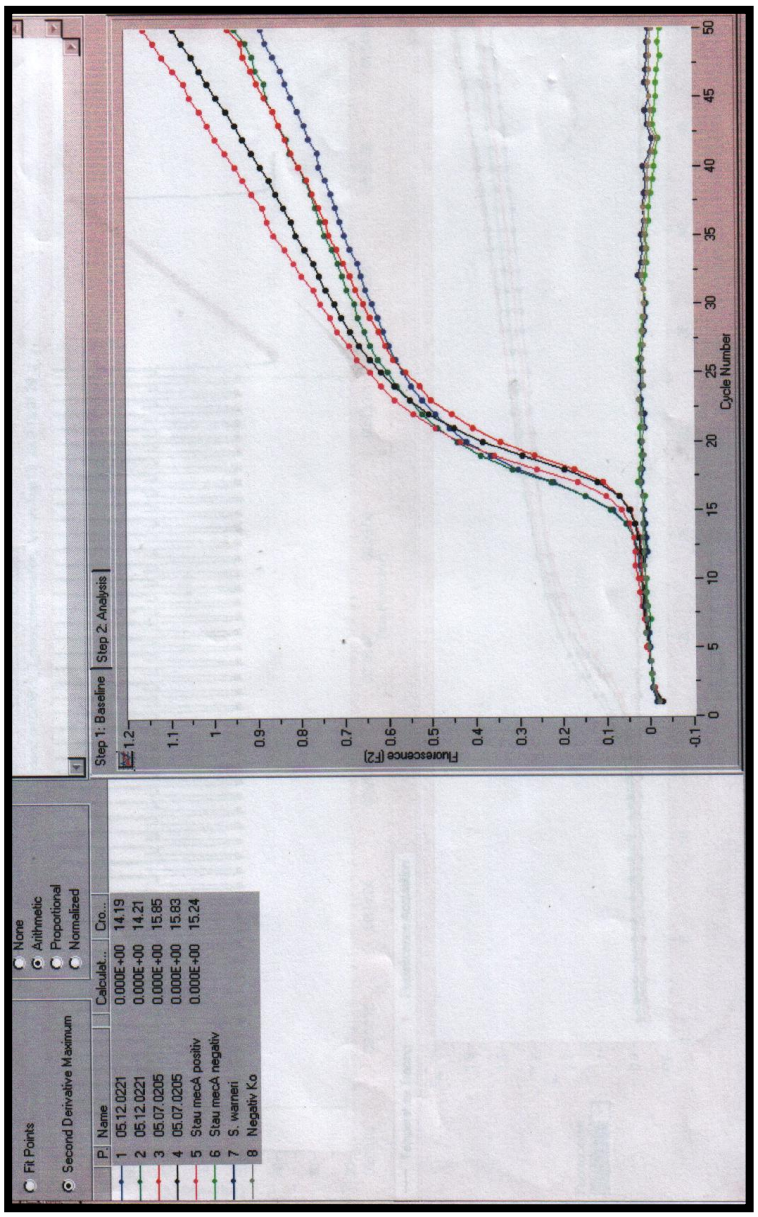

Figure 2: Detection of MRSA DNA with the LightCycler instrument and the LightCycler *Sa442/MecA* Primer/Hybridization probes. Positive results are indicated by upward-deflecting curves as seen here in both the positive control and the positive sample. F2 refers to the fluorescence emission for the LC-Red640. Blank and *S. warneri* were negative controls; MRSA (**pink line**) was the positive control; and samples 1 (**blue line**), 2 (**green line**), 3 (**red line**), and 4 (**black line**) were positive.

4. Discussion

Since the introduction of semisynthetic penicillins, such as methicillin and oxacillin, for the therapy of infections caused by *S. aureus*, the occurrence of *S. aureus* strains resistant to methicillin has steadily increased and MRSA strains have become major nosocomial pathogens ***(Voss et al., 1994)***. The emergence of MRSA is of great concern, as MRSA strains are often multidrug resistant ***(Chambers, 2001).*** Infections with MRSA are known to be associated with considerable morbidity and mortality ***(Cosgrove et al., 2003).*** Many studies have shown that effective control measures, including the systematic screening of persons exposed to MRSA, can confine or even eliminate the nosocomial spread of MRSA ***(Kotilainen et al., 2003).*** The present study was carried out in Sohag University Hospital during the period from September 2011 to February 2012. One hundred nasal swabs were collected from newly admitted patients to different wards of Sohag University Hospital (65 males and 35 females); with age range from (25 to 65 years). In this study we used both conventional culture methods and duplex real time PCR to detect MRSA nasal colonisation. Nasal screening by both methods of

detection identified 26 (26 %) MRSA carriers and 74 (74%) were negative for MRSA nasal colonization. MRSA nasal colonization was more in males 15 (57.7%) than females 11 (42.3%). This finding is more than that reported by ***Al-Rawahi et al. (2008);*** (19%). However it is less than that reported by ***Atkinson et al. (2009);*** (49%). In the current study we used standard culture methods for the identification of *S. aureus* and the determination of oxacillin susceptibility which are time-consuming, which required 2 to 4 days. For these reasons, it has become important to develop rapid diagnostic tests for the detection of MRSA for more active surveillance and control of MRSA to reduce the medical and economic burden from this aggressive pathogen ***(LeDell et al., 2003).***The molecular detection of MRSA directly from clinical specimens containing a mixture of staphylococci, such as screening swabs from anterior nares, represents an important challenge for the rapid detection of MRSA carriers ***(Jonas et al.,2002).*** To overcome this challenge, a multiplex PCR assay have been developed which provides a link between *mecA* and the *S. aureus* chromosome ***(Ito et al.,2001)***. So, in our study we used the duplex real time PCR which is a new molecular assay that targets both an *S. aureus*-specific gene and the *mecA* gene within a single PCR assay for detection of MRSA nasal colonization with the time from the start of processing of specimen to result was approximately three hours; this assay was as effective as the conventional culture methods in detection of MRSA nasal colonization but it was more rapid, sensitive & specific. This assay was able to differentiate methicillin-sensitive *S. aureus* from methicillin-resistant coagulase negative *Staphylococcus* spp. in primary specimens where both of these organisms could coexist in the anterior nares. Because this PCR assay is easy to perform and is rapid, sensitive, and specific ***(Huletsky et al., 2004)***, it should provide a useful alternative for infection-control practitioners to better control the spread of MRSA in our hospitals.

Summary and Conclusion

The rate of methicillin-resistant *Staphylococcus aureus* (MRSA) infection continues to rise in many health care settings. Rapid detection of MRSA colonization followed by appropriate isolation can reduce transmission and infection. Real-time PCR represents a quantum leap in molecular diagnostics. Real-time PCR may rapidly identify MRSA within 3 hours (DNA preparation, amplification, and detection) by the concurrent application of species-specific and *mecA*-specific DNA probes. This study has demonstrated that real-time PCR enables rapid MRSA detection directly from nasal swabs. This testing method combines PCR chemistry with fluorescent probe detection of amplified product in the same reaction vessel so; the risk of releasing amplified nucleic acids into the environment is negligible. The combination of excellent sensitivity and specificity, low contamination risk, and speed has made real-time PCR technology an alternative to culture- or immunoassay-based testing methods for routine screening of nasal carriage of MRSA for our newly admitted patients in our hospitals. In conclusion, real-time PCR test which is equal to or better in sensitivity than culture for detection of MRSA directly from nasal swabs; is rapidly performed (3-hours assay time) and has proved to be sensitive, accurate, and rapid strategy for detection of MRSA nasal colonization, it is the ideal situation to screen all patients admitted to the hospital for preemptive identification of MRSA carriers thus enabling infection control practitioners to identify and efficiently handle these patients according to agreed hospital protocol.

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5. References

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