DETECTION FOR *MECA* GENE IN EGYPTIAN CLINICAL *Staphylococcus aureus* SAMPLES AND STUDY THEIR GENOTOXIC ACTIVITY

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ABSTRACT

Staphylococcus aureus isolates were isolated from fifty clinical samples collected from Ain Shams University Hospitals (March - July 2016) and identified using biochemical and microbiological tests. PCR was performed using specific primers to determine the isolates of Methicillin - Resistant *Staphylococcus aureus* (MRSA) based on the presence of *mecA* gene. Thirty-four isolates from total fifty isolates (68%) were identified as MRSA isolates. To study the genotoxicity for this isolates, forty-five mice were injected with these MRSA isolates and comet and micronuclei assays were performed on mice liver tissues and bone marrow respectively. These assays revealed 24% - 22% DNA damage as an indicator for chromosomal breakage by comet and micronucleus assays respectively which indicate that infection with these isolates leads to mutations. Studying these isolate furthermore will give an insight on how critical maintaining high standard hygiene in Egyptian hospitals and attention to infection control system can prevent occur of outbreaks

Key words: *Staphylococcus aureus*, *MecA* gene, Methicillin-Resistant *Staphylococcus aureus* (MRSA), Polymerase Chain Reaction (PCR), Comet assay, Micronucleus assay

INTRODUCTION

Staphylococcus aureus is an important human pathogen that causes wide range of infection in hospitals and societies including superficial infection and systemic infection. *S. aureus* pathogenicity caused by secreted virulence factors such as extracellular proteins group which facilities binding with the host – in our case – the human **(Gordon and Lowy, 2008)**. *S. aureus* recently became one of the most threatening multi-drug resistant agents, especially in low hygiene hospitals and crowded communities (**Cuervo et al 2010**). Due to its increased prevalence, it developed more than one strain resistant to different antibiotic classes such as Methicillin-which called (MRSA). Methicillin resistant *Staphylococcus aureus* (MRSA) is defined by the integrated of a large transposable element called Staphylococcal Cassette Chromosome mecA (SCCmec) in its genome. That contains the *mecA* gene that encoded to an alternative Penicillin Binding Protein, PBP2a, with low binding affinity to β-lactam antibiotic group **(Foster, 2017).** MRSA developed resistance against Vancomycin which is always has been used to treatment of MRSA infection, this strain called Vancomycin-Resistance *S. aureus* or VRSA strains **(Gradate and Tomasz, 2014)**. In our study, we focused on MRSA pathogen since its furious spread led to increase in the mortality of the patients in hospitals/communities due to the delay of the diagnosis, treatment of the patients, and also the slower response for treatment from patients with weak immunity system such as diabetic and cancer patients **(Priyadharshini et al 2013, Lin et al 2017 and Rozgonyi, 2007)**. MRSA pathogen behaves differently to protect itself from host immunity system. It secretes molecules to escape from innate human immune system by inhibiting the function of phagocytosis and neutrophils. Furthermore, escaping from complement system that participate in protect human from any invasion which lead to genotoxicity (DNA damage) **(McGuinness et al 2016)**. Surprisely, not many studies focused on the genotoxicity that caused by MRSA pathogen even though, the presence of reliable genetic methods to detect

the genotoxicity, such as the Comet and micronucleus assay **(Karyank et al 2015)**.

That's why our study was initiated; to work on detecting and identifying Egyptian MRSA isolates in hospitals and recognize the ability of these isolates to damage the DNA through genotoxicity of the patients. So we can shed some light on the expected approach to prevent the spreading of this MRSA pathogen in hospitals.

MATERIALS AND METHODS

This study was carried out at Ain Shams Center of Genetic Engineering and Biotechnology laboratories (ACGEB) and National Research Center (NRC). Fifty bacterial isolates from various clinical samples including blood, urine, sputum, wounds, pus, ascetic fluid and abscess, were collected from Department of Microbiology, Ain Shams University Hospitals, Cairo, Egypt. Standard *S. aureus* used in this study identified as *S. aureus* standard strain (ATCC 6538) collected from Cairo Microbiological Resources Centre (Cairo *MIRCEN*) in Ain Shams University, Faculty of Agriculture – Shobura – Egypt.

Bacterial Identification

All isolates were identified and confirmed as *S. aureus* based on biochemical tests. It were streaked on Baird Parker as a selective medium and incubated at 37°C for 24 hrs. as well, stained by Gram stain, Catalase and coagulase tests were also performed **(Mossel, 1962 and Costa, 2013).**

Polymerase chain reaction for the detection of *mecA* gene in *Staphylococcus aureus* isolates

Chromosomal DNA was isolated from each *S. aureus* isolates using the DNA isolation kit (Promega, USA), the quality of the chromosomal DNA was evaluated by concentrations were determined using Quantus Fluorometer (Promega, USA) by QuantiFluor® dsDNA System Kit, and normalized to 10 ng/µl. PCR was performed on all *S. aureus* isolates using PCR master mix (Promega, USA) to determine the presence of *Mec A* gene, by specific primer **(Geha et al 1994 and Pillai et al 2012).** The oligonucleotides sequence of the 25-bp primer pair used as follow: *mecA1* F: 5′-GTAGAAATGACTGAACGTCCGATAA-3` (318-342) and *mecA2* R: 5′-CCAATTCCACATTGTTTCGGTCTAA-3` (603-627) which produce 310 bp PCR products. The components of each PCR 25µl reaction: PCR Master Mix, 12.5µl; each primer 1µM; template DNA (3.6-9.8) µl according to DNA concentration for each sample, complete with water nuclease-free up to 25µl as total volume. **PCR program** included 30 cycle of pre-denaturation at 94ºC for 4 min. followed by denaturation at 94ºC for 45 sec., annealing at 55ºC for 45 sec., extension at 72ºC for 1 min., and final extension for 5 min.

Genotoxicity assay

This part from the study was approved by the Ethical Committee of Animal Research (ECAR) at National Research Center (NRC). Forty five albino males' mice were used, six weeks of age, weighing approximately 25 g, divided into nine groups, each group had five animals as replicates, and were injected interproteneal by different types of samples from wound, sputum, ascetic fluid, urine, blood, pus isolates and MSSA strain as a negative control.

Preparation of bacterial inoculums for mice injection

The colonies were grown on Trypticase Soy Broth (TSB) medium **(Silva-Santana et al 2016)** for 24 hrs. to acquire the dilution of 1*106 CFU/ml, using tubes containing serial dilutions in sterile saline (0.9% NaCl). The Animals were injected with 100 µl of bacterial suspension, except the control group, which were injected with 100 µl sterile saline (0.9% NaCl) only. Mice were sacrificed 48 hrs. after injection and liver tissue was taken for comet assay and bone marrow was taken for micronucleus assay.

Comet assay

Liver tissues were digested in trypsin to produce a single cell suspension; cells were placed on a microscope slide coated with agarose gel. The slide was then placed in solution containing 0.5% SDS, 30 mM EDTA, p^H 8.0 at 50°C for 4 hrs. then the slides were left overnight at room temperature in Tris/Borate/EDTA buffer, p^H 8.0. Samples were electrophoresed for 25 min. at 0.6 v/cm, and then stained with Ethidium Bromide. Images by fluorescent microscope with a CCD camera were taken and count the cells which took the comet shape to determine the percentage of cells with DNA damage in 100 cells for each sample. **(Olive et al 1990, Maynou et al 2014 and Collins et al 1997)**.

Micronucleus assay

The bone marrow cells were left in small volume of fetal calf serum for slide preparation and fixation by methyl alcohol and stained by 5% Giemsa stain for 10 min., prepare three slide for the same animal, under microscope and search on the percentage of Micronucleated PolyChromatic Erythrocytes (%MnPCEs) Between 2,000 polychromatic erythrocytes (PCEs) under oily lens **(Adler, 1984 and Alad, 2009)**.

Statistical analysis

Statistical analysis was performed using SPSS software program (10.0) (1998 – by SAS Institute Inc., Cary, NC., USA), The PCR results were statistically analyzed by Chi-square analysis as well as calculated variables as percentage followed by ANOVA one way analysis. As for the comet and micronucleus assay parameters, the critical level for rejection of the null hypothesis was considered as less than 5% is significant and more than 5% non-significant.

RESULTS AND DISCUSSION

Fifty isolates were collected from males and females equally, different ages including infants and adults, also from different clinical samples (urine, abscess, blood, pus, wound, sputum and ascetic fluid).

Identification of the isolates was performed using biochemical tests. All isolates were cultured on Baird Parker medium as selective media for *S. aureus* giving black and shiny colonies as shows in **Fig. 1**, staining with Gram stain and all isolates gave Gram positive results as shows in **Fig. 2**, also, Catalase test was performed to all isolates which gave Catalase positive results as shows in **Fig. 3**. All isolates were confirmed to be *Staphylococcus.*

Besides, as the pie chart in **Fig. 4** shows the percentages of *Staphylococcus* presence in different types of the clinical samples collected from the hospital. Out of 50 samples, 35% were wound samples, 29% were blood samples, 14% were pus samples, 10% were sputum samples, 6% were abscess samples, 4% were urine samples, and 2% were from ascetic fluids.
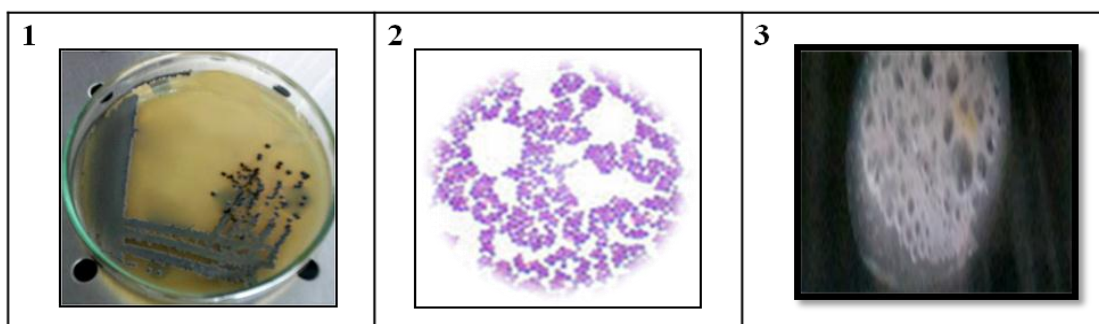

Fig. 1. *S. aureus* growth on Baird Parker medium

Fig. 2. *S. aureus* Gram stain +
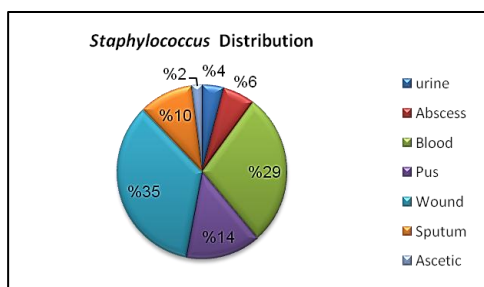
Fig. 3. *S. aureus* Catalase +

Fig. 4. Distribution of % Staphylococcus isolates from different clinical samples

Furthermore, the detection of MRSA isolates prevalence in the fifty isolates from the clinical samples was performed using PCR specific primer for *mecA* gene with product size of 310 bp. MRSA isolates represented 34 isolates from the total isolates while the MSSA isolates represented the latter of the isolates as shown in **Fig. 5**.
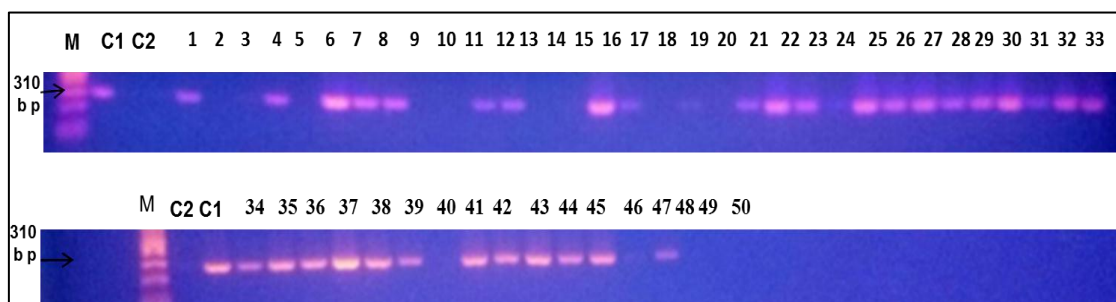
Fig. 5. *MecA* gene detection within the *Staphylococcus* isolates from clinical samples; C1: *S. aureus* ATCC 6538 (MSSA strain- negative control); C2: *S. aureus* (MRSA strain- positive control); and M: 100bp DNA ladder

As shown in **Table 1**, PCR results recorded MRSA isolates were represented by 68%, whereas MSSA isolates were represented 32%. There was significant difference between MRSA and MSSA prevalence (p <0.05).

Table 1. Distribution of MRSA/MSSA isolates from different types of samples detected by PCR

| Type of Sample	MSSA Isolates		MRSA Isolates	
	Number/ Total	%	Number/Total	%
Blood	6/50	10%	8/50	18%
Pus	1/50	2%	6/50	12%
Urine	-	--	2/50	4%
Sputum	1/50	2%	4/50	8%
Ascetic Fluid	-	--	1/50	2%
Abscess	1/50	2%	2/50	4%
Wound	6/50	12%	11/50	20%
S. aureus Reference Strain	1/50	2%	-	-

After MRSA isolates were detected, each group of mice was injected with different type of samples from these isolates **(Table 2)**. Tissues from bone marrow and liver samples were collected from mice, and a significant change was observed in the shape and color of the liver as shown in **Figure 6**. Infected liver samples were used in the comet assay and bone marrow samples were used in micronucleus assay.

Fig. 6. Comparison between normal liver tissue and infected liver tissue with MRSA isolate

As for the Comet assay as a genotoxicity assay, visual damage in the DNA caused by the MRSA infection was noticed and documented as shown in **(Figure 7)**. It showed three different levels of damage (class1, 2 and 3) in DNA in all infected mice liver compared to the healthy ones (class 0).
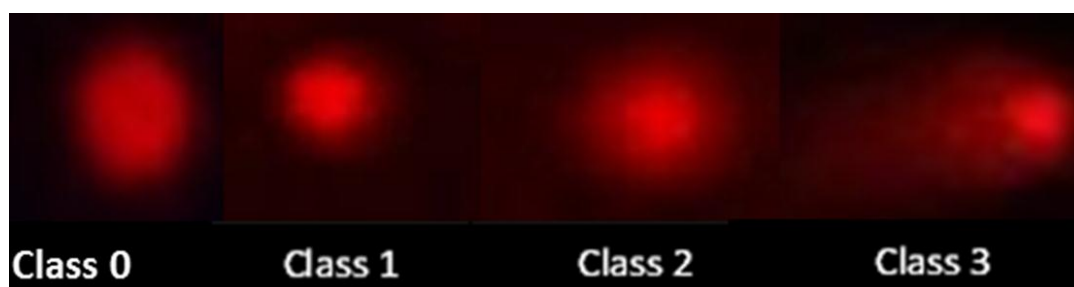
Fig. 7. Classification of fluorescent images of the Comet assay: Normal nucleus (class 0), gradual increase in the comet tail (class 1), with a decrease in the nuclear DNA content (class 2 and class 3)

Also, 500 cells from each mouse were investigated and classified according to type of class of damage (class 1, 2 and 3) and this visual damage of DNA was scored as shown in **Table 2**.

There were significant differences between DNA damage that occurred in MRSA isolates from pus, wound, ascetic fluid, urine, blood and sputum samples compared to no DNA damage in MSSA strain (ATCC 6538) as a negative control. **Furthermore,** the mean of the percentage of DNA damage in comet tail which occur in all MRSA isolates, was calculated and compared to the percentage of no DNA damage in comet tail of all control groups as shown in **Table 3.**

Table 2. Visual score of DNA damage in mice liver tissues treated with MRSA isolates collected from the clinical samples (mean ± SD)

Treatment	No. of cells		Class				SD	DNA damaged cells % (Mean±SEM)
	Analyzed[*]	Comets	0	1	2	3		
Saline (- control)	500	26	474	21	5	0	0.75	5.2±0.75 [d]
MRSA (+ control)	500	152	348	41	52	59	1.62	30.4±1.62[a]
MRSA from Sputum	500	91	409	33	35	23	1.47	18.2±1.47 [c]
MRSA from Wound	500	113	387	34	41	38	1.50	22.6±1.50[b]
MRSA from Ascetic fluid	500	103	397	40	29	34	1.50	20.6±1.49[bc]
MRSA from Urine	500	85	415	26	32	27	1.41	17.0±1.41[c]
MRSA from Blood	500	107	393	35	33	39	0.80	21.4±0.80[bc]
MRSA from Pus	500	116	384	39	41	36	1.47	23.2±1.47[b]
MSSA (- control)	500	98	402	31	38	29	1.02	19.6±1.01[c]

a,b,c,d, represented significant difference between groups

Table 3. Comet parameters for analyzed 500 cells from each mouse's liver (Mean ± SEM)

Comet parameters	Mice injected with MRSA strain (n=35) (Mean±SEM)	Mice injected with MSSA strain (n=5) (Mean±SEM)	Controls (n= 5) (Mean±SEM)
Percentage of DNA damage in comet tail	23.76±1.525	19.6±1.01	5.2±0.75
Percentage of DNA damage in comet head	76.24±1.525	80.4±1.01	94.8 ±0.75

SEM: standard error

Subsequent to the comet assay, micronucleus assay, as a confirmation assay, was performed by screening 2000 polychromatic erythrocytes cells with three replicates in all bone marrow cells **(Figure 8).** The mean of Micronuclei (Mn) in Poly-Chromatic Erythrocytes (PCE) from MRSA-infected bone marrow cells was calculated and found statistically significant in all treated groups compared to non-treated groups – NormoChromatic Erythrocytes (NCE) as negative controls as shown in **Table 4**.
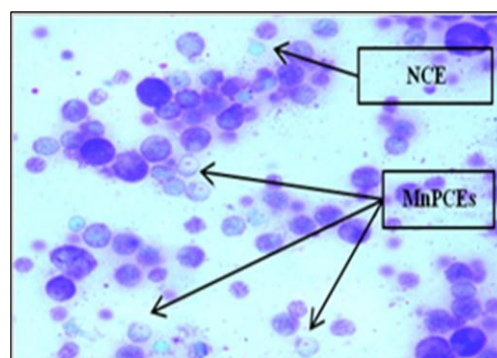
Fig. 8. Bone marrow cells under light microscope – untreated - NormoChromatic Erythrocytes (NCEs) and MRSA-treated - Micronucleated PolyChromatic Erythrocytes (MnPCEs)

Table 4. The treatments of mice with different MRSA and control samples and MnPCEs and NCEs in bone marrow cells (mean ± SD).

Treatment (mg/kg)	PCE screened	MnPCEs/2000 PCE			NCE		PCE/NCE	
		Number	Mean	SD	screened	Ratio	Mean	SD
Saline (- control)	2000	7	6.33	1.15	743	2.69	2.71	0.05
	2000	5			752	2.66		
	2000	7			723	2.77		
MRSA (+ control)	2000	33	29.33	3.51	542	3.69	3.42	0.24
	2000	26			598	3.34		
	2000	29			619	3.23		
MRSA from Sputum	2000	21	19.67	1.15	663	3.02	3.15	0.11
	2000	19			621	3.22		
	2000	19			623	3.21		
MRSA from Wound	2000	25	23.33	1.53	573	3.49	3.31	0.24
	2000	23			588	3.40		
	2000	22			657	3.04		
MRSA from Ascetic Fluid	2000	23	21.67	1.15	673	2.97	3.01	0.36
	2000	21			750	2.67		
	2000	21			591	3.38		
MRSA from Urine	2000	18	18.67	0.58	688	2.91	2.96	0.11
	2000	19			649	3.08		
	2000	19			695	2.88		
MRSA from Blood	2000	23	22.67	1.53	643	3.11	3.20	0.17
	2000	24			649	3.08		
	2000	21			589	3.40		
MRSA from Pus	2000	25	24.67	1.53	681	2.94	3.23	0.27
	2000	26			611	3.27		
	2000	23			577	3.47		
MSSA (- control)	2000	23	20.67	2.08	633	3.16	3.12	0.05
	2000	20			654	3.06		
	2000	19			639	3.13		

DISCUSSION

In recent years and due to low hygiene in hospitals, MRSA strains became widely spread in Egypt. There were several studies documented the appearance of MRSA strains in hospitals through Egypt. In our study and out of 50 isolates, 68% of them were MRSA isolates which were found between patients in Ain Shams University Hospital. In similar study carried out by **Hefzy and Hassan (2016)** out of 70 *S. aureus* isolates collected from Fayoum University Hospital, 57% of them were MRSA isolates, while in Mansoura University Hospital and out of 70 *S. aureus* isolates collected, there were 40% of these isolates represented MRSA **(Elshabrawy et al 2017)**. Each study has been done in Egypt focused on the detection of *mecA* gene and finding the perfect matched primer pairs that cover big portion of the gene, which known for its responsibility of inducing resistant for β-lactam group triggering different symptoms of MRSA infection in hospitalized patients. So for

Hefzy and Hassan (2016), they used duplex PCR to identify *mecA* gene, but in **Elshabrawy et al 2017**, they used primers to search for the whole cassette of *mecA* gene (SCCmec). In our study, we used a very specific primer that has been used in **Pillai et al 2012**, which was able to detect 37% MRSA isolates from 165 samples collected from different hospitals in India. The same primer pairs was used by **Sahebnasagh et al 2014** in Iran to successfully identify 69% MRSA isolates from 100 samples. All these primer pairs were actually designed and adjusted to pick *mecA* gene through multiplex PCR which was done by **Geha et al 1994** who successes to detect 17% MRSA isolates from 228 samples collected from different states in U.S.

Furthermore and due to the increase in lethality of MRSA pathogen around the world between adults, it was actually causing more death cases in neonates especially in the United States (**Gordon and Lowy 2008**).That is why our study on MRSA pathogen focused on detecting genotoxicity in the hospitalized patients. We found that using comet assay is considered the most reliable assay to detect the genotoxicity through capturing the DNA damage caused by MRSA pathogen even though not previous studies on comet assay were performed before in Egypt. Comet assay was performed in our study on different samples of MRSA isolates and the result showed significant differences before and after the treatment that caused genotoxicity. Three classes of damage were seen under the fluorescent microscope, class 1 was presented by simple DNA damage, class 2 was represented by moderate DNA damage, and class 3 was represented by severe DNA damage. But in other countries such as in India, **Priyadharshini et al 2013,** MRSA isolates led to septicemia in the neonates which urged the detection of genotoxicity through using comet assay and it confirmed the three different classes of DNA damage.

In addition to comet assay, micronucleus assay was performed to confirm comet assay results. There were significant differences in DNA damage between control groups and all mice groups which were injected with MRSA isolates. This indicated the ability of MRSA isolates to cause mutagenicity **(Priyad harshini, et al 2013 and El-Gendy et al 2017).** A similar study in Egypt was performed by **El-Gendy et al (2017)** using rats as model organism and injected it with six different MRSA isolates. These isolates were isolated from patients with cancer and the micronucleus assay was performed. Micronucleated cells were calculated and evaluated and the results were similar to our re-sults which indicate the severe damage that MRSA pathogen can cause.

mecA gene is a marker for MRSA strains detection by PCR, MRSA strain became more virulent strain around the world and keep increasing in prevalent, and after use many reliable genetic methods such as comet and micronucleus assay, we can say that *S .aureus* has ability to induce genetic toxicity in hospitalized patients

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الكشف عن جين الـ MecA في عزلات البكتريا العنقودية المصرية ودراسة السمية الوراثية الخاصة بها

[49]

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الموجـــــــز

تم جمع خمسون عينة إكلينيكية من مستشفيات جامعة عين شمس خلال الفترة من (مارس– يوليو 2016)، والتي تم تعريفها بإستخدام اختبارات كيميائية حيوية وميكروبيولوجيه لعزل بكتيريا *S. aureus*. وتم إجراء تفاعل البلمرة المتسلسل باستخدام بادئات محددة لتحديد عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA) على أساس وجود الجين *mec A*. وتم تحديد 68٪ من هذه العزلات بأنها MRSA. وتمت دراسة السمية الوراثية لهذه العزلات على نخاع العظام ونسيج كبد 45 من فئران التجارب، باستخدام

مقاييساتmicronucleus test، comet assay التي كشفت عن ضرر الحمض النووي بنسبة 22٪– 24٪ كمؤشر لتكسر الكروموسومات على التوالي والتي تشير إلى أن العدوى بهذه العزلات تؤدي إلى طفرات. وعلاوة على ذلك، فإن دراسة هذه العزلات ستعطي فكرة عن ضرورة الاهتمام بنظام مكافحة العدوى في المستشفيات المصرية لمنع حدوث انتشاره هذا النوع من البكتيريا.

الكلمات الدالة: البكتريا الذهبية العنقودية، جين الـ MecA، البكتريا الذهبية العنقودية المقاومة للميثيثلين، تفاعل البلمرة المتسلسل، اختبار الـ Comet، إختبار الـ Micronucleus