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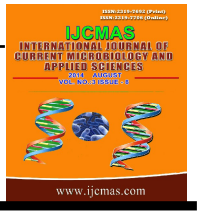

Original Research Article

Incidence of postoperative endophthalmitis and the potential of marine sponges as new sources of antibiotics in Egypt

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A B S T R A C T

To identify microbial pathogens in aqueous humor of patients with postoperative endophthalmitis using polymerase chain reaction (PCR) and conventional methods and studying antimicrobial activity of marine-derived bio products. Fifty patients clinically diagnosed as postoperative endophthalmitis, mainly after cataract surgery, who attended the Research Institute of Ophthalmology were investigated in our study. A total of fifty persons were used as controls. Conventional methods, including direct microscopy and culture, and PCR were used. Antibacterial activity of crude extracts from sponges against microbial strains was determined by the agar-diffusion method. Among the fifty aqueous humor samples, 28% showed positive culture growth and 60% were PCR positive. When associated, culture and PCR allowed for a microbiological diagnosis in 76% of cases. Microorganisms cultured by conventional techniques matched those identified by PCR. The most common isolated microorganism was *Pseudomonas aeruginosa* (57%) followed by *Staphylococcus aureus* (43%). Aqueous and ethanolic extracts of sponge species showed inhibitory activity against *Staphylococcus aureus* more than *Pseudomonas aeruginosa*. Detection of microbial DNA by PCR may prove a useful and rapid means of diagnosing postoperative endophthalmitis and facilitating management decisions when conventional culture is negative. Marine sponges are among the most promising sources of new antimicrobial substances.

Keywords

Antimicrobial activity - aqueous humor - Egypt - p c r- postoperative endophthalmitis

Introduction

Postoperative endophthalmitis remains a serious clinical problem in ophthalmology. Postoperative endophthalmitis after cataract surgery is one of the major devastating complications of intra-ocular surgery [1,2]. Its occurrence can lead to poor visual prognosis, and increase in health care cost. Postoperative endophthalmitis following cataract surgery is the commonest form of endophthalmitis, accounting for approximately 70 per cent of infective endophthalmitis [3]. It is defined as

intraocular inflammation caused by an infective process following intraocular surgery, where the full thickness of the cornea and / or sclera has been breached. This definition excludes patients with penetrating eye injury. The source of infection following intraocular surgery include lids, adnexa, ocular tear film, respiratory and skin flora of the surgeons and assistants, surgical instruments including irrigating solutions and operating room air [4]. Acute-onset postoperative endophthalmitis (AOPE) occurs most frequently after cataract surgery, the most common intraocular procedure, with an incidence of ~0.05%. AOPE is more common after secondary intraocular lens implantation (0.20% of cases) and less common after virectomy [5]. Chronic postoperative endophthalmitis (CPE) may present with nonspecific inflammatory signs weeks or months following surgery. The patient may have mild cells and flare in the anterior chamber, vitreous inflammation, and/or cystoid macular edema. Frequently, these entities are responsive to corticosteroid treatment. *Propioni bacterium acnes* is the most commonly isolated organism in cases of CPE but many other bacteria, mostly with low virulence, have been implicated in the syndrome [6]. The potential of marine organisms as a source of new substances is huge. Marine species comprise approximately half of the total global biodiversity [7].

Marine sponges (phylum Porifera) are among the most ancient multicellular animals (metazoans). These sessile, filter feeding animals are a rich source of novel biologically active metabolites and offer great potential for drug discovery and, in the long term, for treatment of cancer and infectious diseases [8]. The increasing prevalence of multi-resistant bacteria made the search of new antibacterial agents an important strategy for the establishment of alternative therapies in difficult handling infections [9]. Sponges of the Red Sea offer a potential for production of novel drugs and prototypes. The genus *Negombata* (class Demospon-giae, order Poecilosclerida, family Podospongiidae) is a type of sponges abundant in the Red Sea. This sponge produces latrunculins that have well documented antitumor activity in addition to antimicrobial and antiviral effects. The genus *Negombata* is represented in the Red Sea by two species, namely *Negombata magnifica* (Keller) (formerly *Latrunculia magnifica)* and *Negombata corticata* (Carter) [10].

Materials and Methods

Fifty patients clinically diagnosed as postoperative endophthalmitis, mainly after cataract surgery, who attended the Research Institute of Ophthalmology were investigated in our study. Control group included fifty healthy persons undergoing cataract surgery under the same preoperative circumstances as the patient group.

Samples

Aqueous fluid was collected from both groups. The samples were divided in two parts: one part stored at -70°C for polymerase chain reaction (PCR) evaluation, another part was used immediately for conventional microbiological diagnosis.

Conventional microbiological diagnostic techniques

The specimens were processed on the same day of collection. Direct microscopy was performed on 10% potassium hydroxide (KOH) wet mount and Gram staining under light microscope for detection of bacteria or yeast. Also the samples were inoculated directly onto sheep's blood agar, chocolate

agar, Sabouraud dextrose agar (SDA) and Wilikins Chalgren anaerobic agar. Deep inoculation in brain heart infusion (BHI) broth was also done. The inoculated media were incubated at 37°C, examined daily, and discarded after 5 days if no growth was observed. The inoculated Sabouraud dextrose agar was incubated at 27°C, examined daily and discarded after 14 days if no growth was detected while Wilikins Chalgren anaerobic agar was incubated anaerobically in anaerobic jar at 37°C and examined after 5 days. Following adequate growth of the fungal isolates on Sabouraud dextrose agar, identification was done based on its macroscopic and microscopic features using lactophenol cotton blue stain solution. If positive bacterial growth was obtained on the different culture media, the standard biochemical tests were performed and further identification was done up to the species levels using the API STAPH and API 20 NE systems.

Polymerase Chain Reaction (PCR)

Sample collection and DNA extraction

DNA from all samples was extracted within one month of receipt. Briefly, DNA was extracted from each swab using QIA amp DNA Micro extraction kit from Qiagen according to manufacturer's instructions. QIA shredder was also used to harvest the lysate.

DNA amplification

The primers used in this study, their sequence, product size and references are shown in Table 1. Specificity of the primers was tested using DNA of various strains available in our microbiology and immunology laboratory. All the strains used as positive controls were laboratory isolates like *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Candida* spp., *Aspergillus* spp. and *Fusarium* spp.

Conditions for bacterial universal primer

16srRNA primers (For. 27-6 and Rev. 28-6) were dissolved in 165 µl dist. water to reach final conc. 50 pmole/µl, product size 241 bp.

PCR reaction mix (50µl)

The mix contained DNA 1µl, taq 0.25µl, primers (pF 0.5 + pR 0.5), 5x buffer (GoTaq Reaction buffer) 10 µl, dNTPs 4 µl (2 mM) and complete with dist. Water 33.75 µl.

PCR program:

Initial denaturation 96 ^{o}C for 3 min., denaturation 95^{o}C for 15 sec., both extension and annealing in one step 55^{o}C for 30sec., 40 replication cycles, final extension 55^{o}C for 10 min., stop reaction at 4 ^{o}C for 10 min.

Conditions for the bacterial species analysed

(*Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas* spp.)

PCR reaction mix (50µl) for bacterial genotyping

The mix contained DNA 4µl, taq 0.5µl, 5x flexi buffer 10µl, dNTPs 1µl (10mM), Mg 7.5µl for *S.aureus* and 6.5µl for *S. epidermidis* and *pseudomonas*, primers (0.3For +0.3Rev) complete with dist. water.

Staphylococcus aureus

By using Forward primer (9-6) and Reverse primer (10-6), product size 477 bp. (9-6 dissolved in 226µl and 10-6 dissolved in 240µl dist. water).

PCR program

Initial denaturation 95^{o}C for 5 min., denaturation at 95^{o}C for 10 sec., annealing 60^{o}C for 10 sec., extension 72^{o}C for 22 sec.45 cycles of replication and final extension 72^{o}C for 5 min.

Staphylococcus epidermidis

By using GYR (Gyrase)b For-9 and GyRb Rev-9 primers, product size 251 bp. (GYRb For-9 dissolved in 188µl and GYRb Rev-9 dissolved 185µl dist. water).

PCR program

Initial denaturation 95^{o}C for 5 min., denaturation step at 94^{o}C for 30 sec., annealing 55^{o}C for 30 sec., extension at 72^{o}C for 1min., 40 cycles of replication and final extension at 72^{o}C for 2 min.

***Pseudomonas* spp.**

By using For. 21-6 and Rev. 22-6 primers, product size 618 bp**.** (21-6 dissolved in 185µl and 22-6 dissolved in 205µl dist. water)**.**

PCR program

Initial denaturation 95□C for 2 min., denaturation 94^{o}C for 20 sec., annealing at 51^{o}C for 20 sec., extension at 72^{o}C for 40 sec., 40 replication cycles and final extension at 72^{o}C for 1 min.

- PCR product was run on 1.5% agarose gel for medium product size (>400 bp) and 2% agarose for small products size (<250 bp), samples run with 100pb ladder.
- Electrophoresis voltage range from 100:200 v, depend on the size of the gel. Small gels (50 ml) run on 100:120 V, large gels (100ml) run on ≈150 V.

Sponge collection and sample processing

Specimens of the Red Sea sponge *Negombata* were collected by SCUBA diving at two different locations in the Red Sea; Ras Mohamed and Safaga, Egypt at a water depth of 10 m. *Negombata magnifica* was collected from Ras Mohammed while *Negombata corticata* was collected from Safaga.

Sponge samples were cut with a dive knife while wearing latex gloves and individual pieces were put into separate plastic sample collection bags. Samples were brought to the surface, maintained at ambient sea water temperature, and transported to the lab for processing. A section of the sponges specimens was frozen on dry ice and stored at −70°C. Fragments of collected specimens were stored in 70% ethanol for morphological characterization and taxonomic classification.

After collection, the specimens were processed as follows: 5–10 g of sponge tissue was macerated in sterile distilled water or ethanol to obtain crude extract of each at a final concentration of approximately 1.0 g/ml. Aqueous extract and ethanolic extract were centrifuged at 5000 x g for 5 min and the supernatant were filtered (Millipore 0.22 mm) and stored at 4 °C.

Taxonomical classification

The collected samples of the sponge material were morphologically identified and taxonomically classified at the Marine Biology Department, El Gouna, American University in Cairo. The identification of *Negombata* species is based on morphology and microscopical examination of megascleres of spicules.

Assay for antibacterial activity

Antibacterial activity of crude extracts from sponges against tested strains was determined by the agar-diffusion method as described by Marinho *et al*. (2010) [15]. Briefly, 20 µl of the crude extracts were spotted on BHI agar. The test organisms were previously grown in BHI medium at 37 °C for 18 h. Approximately 10^5 cells were suspended in 4 ml BHI soft agar and poured over the plates, which were subsequently incubated at 37 °C. After 18 h, the inhibition zones around the spotted material were observed. Inhibition zones >8 mm were considered indicative of inhibitory activity [15].

The protocol for sample collection was in accordance with the routine work conducted in the Research Institute of Ophthalmology pertaining to the conventional preoperative preparations for any cataract surgery patient admitted for operation.

Results and Discussion

Culture and smear results

Among the fifty patient samples, 24 (48%) were positive for direct microscopy by Gram stain and 14 (28%) were positive by culture. All 14 positive culture cases were also positive by direct microscopy. The predominant organisms detected in the 24 positive direct microscopy patient samples were *Pseudomonas aeruginosa* in 14 patients and *Staphylococcus aureus* in 10 patients; while in the 14 positive culture patients, *Pseudomonas aeruginosa* was isolated from 8 patients and *Staphylococcus aureus* isolated from 6 patients. Consequently, all 14 positive culture and direct microscopy patients had *Pseudomonas aeruginosa* and *Staphylococcus aureus* in 8 and 6 patients respectively. Results are shown in table 2.

Polymerase chain reaction and culture results

Among the fifty patient samples, 14 (28%) showed positive growth in culture (all bacterial); and 30 (60%) were positive by PCR. Out of the 14 positive culture cases, 8 were also positive by PCR and the remaining 6 cases were PCR negative. When associated, culture and PCR of aqueous humor samples allowed for a microbiological diagnosis in 76% (38) of cases. Microorganisms cultured by conventional techniques matched those identified by PCR. Among the 30 PCR positive cases, 18 (60%) were *Pseudomonas aeruginosa* and 12 (40%) were *Staphylococcus aureus*. Results are presented in table 3, and figures 1 & 2.

Antibacterial activity of sponge extract

The results of initial screening using aqueous and ethanolic extracts of sponge species showed that they possessed inhibitory activity against *Staphylococcus aureus* more than *Pseudomonas aeruginosa*, where all 6 (43%) *Staphylococcus aureus* culture positive samples were sensitive to the extracts, while only 4 (29%) of 8 *Pseudomonas aeruginosa* culture positive samples were sensitive to the extracts. Inhibition zones varied between 8 and 25 mm.

Bacterial endophthalmitis, with an estimated incidence following cataract surgery of between 0.07% and 0.3% [16, 17], is among the most feared complications of intraocular surgery and may result in severe vision loss [16, 18, 19, 20]. In studies based on conventional culture techniques, coagulase-negative *staphylococci* (CNS) (e.g., *Staphylococcus epidermidis*) account for

nearly 60% of all cases, and *Staphylococcus aureus* accounts for another 20% of the total. While in our study, *Staphylococcus aureus* accounts for 12% of the total 50 patients.

Pseudomonas aeruginosa is a leading pathogen amongst the Gram-negative bacteria causing endophthalmitis. *Pseudomonas aeruginosa* endophthalmitis is a well described syndrome characterized by rapid progression and poor visual prognosis [21]. Although *Pseudomonas aeruginosa* endophthalmitis can occur secondary to penetrating ocular trauma, perforating corneal ulcer or septicaemia, it is noted quite frequently following ocular surgery [21, 22]. Outbreaks of post-operative endophthalmitis due to *Pseudomonas aeruginosa* were reported in the past, in which the source of infection could be traced to the use of irrigating saline, intra-operative use of contaminated ophthalmic solutions or to the implantation of contaminated lenses [23-26].

Direct microscopy by Gram stain provides a rapid diagnosis for bacterial endophthalmitis though it is less sensitive. Microbial culture remains the gold standard in the diagnosis but it takes longer time due to slow growing nature of the organism and should be carefully examined. The possible reasons for low sensitivity in conventional methods include small volume of sample, less organism load in the ocular specimen and a greater tendency of the organisms to be loculated rather than evenly distributed. This prompted us to evaluate PCR technique in diagnosis of postoperative endophthalmitis. Species-specific primers were used for amplification of DNA [27, 28, 29, 30].

The PCR is a powerful technique for amplifying infinite quantities of nucleic acids for further analysis. PCR is an extremely sensitive technique able to detect single copies of pathogen DNA in complex mixtures. PCR has been successfully applied to the diagnosis of many ocular conditions [31]. The availability of DNA primer sets that effectively recognize all bacteria or all fungi suggests this technique may have utility for diagnosis of endophthalmitis.

In our study, among the fifty patient samples, 24 (48%) were positive for direct microscopy by Gram stain, where fourteen samples (58%) were *Pseudomonas aeruginosa* and ten (42%) were *Staphylococcus aureus*. In this study, from the fifty patient aqueous humor samples, 14 (28%) showed positive growth in culture (all bacterial) and 30 (60%) were polymerase chain reaction positive. When associated, culture and PCR of aqueous humor samples allowed for a microbiological diagnosis in 76% (38) of cases. The most common isolated microorganism was *Pseudomonas aeruginosa* (57%) followed by *Staphylococcus aureus* (43%). Among the 30 PCR positive cases, 18 (60%) were *Pseudomonas aeruginosa* and 12 (40%) were *Staphylococcus aureus*.

Sensitivity of the PCR described in this study was higher compared to conventional techniques. The increased sensitivity of PCR over conventional techniques was reported by other studies, as well [32, 33, 34]. Besides, PCR for diagnosis of endophthalmitis was rapid in comparison to culture method but the cost is expensive comparable to conventional methods. Polymerase chain reaction results in this study seemed quite promising. However, the disparity between culture and PCR results may be explained by the fact that the culture positivity requires viable organisms, whereas a PCR-based test can detect both viable and nonviable organisms. PCR test can theoretically be positive even if only a

single copy of target DNA is present. The high positivity of PCR in already treated cases in comparison to culture, reiterates the difficulty in getting a positive culture from noviable organisms in the sample.

In another study, Anand *et al.* [32] highlighted the benefit of time factor in diagnosing fungal endophthalmitis. Although their PCR assay produced results in 8 hours, culture confirmation took almost 10 days. Our study was thus very much similar to theirs because the PCR method used by us yielded results in 4 to 8 hours, depending on the number of cycles repeated. This is a major advantage of the technique, especially when compared to culture where it took at least 5 to 7 days for a positive growth in our setup.

Although various advantages have been attributed to PCR due to its rapidity and widespread applicability to bacteria and fungi, the technique has various reported complexities and drawbacks, as evidenced from our study as well. Some of the limitations are logistic and some technical. Among them is the difficulty in optimization, apart from the difficulty in differentiating between active and latent infections, viable, and nonviable cells. Moreover, the DNA sequence has to be known in advance, and the high sensitivity could lead to false-positive results. Presently, with the everyday discovery and updating of novel lab techniques, the need for widening the limitations of the conventional gold standard-culture is imperative. It would seem logic that the PCR method would be the most ideal to be chosen over the various limitation of culture. Yet one cannot totally ignore the roll of culture which represents a 100% confirmatory result if positive and the direct means of reaching an accurate diagnosis and the initiation of the proper treatment. On the other hand, although PCR technique is a procedure which requires a well equipped lab, personell with experience and standardization, not to mention the cost when compared to culture, yet all these factors when weighed against the time factor needed to reach a rapid diagnosis for the prompt start of therapy that could save a patient's eye from visual loss as in the patients of our study, would fairly favour the PCR technique. Thus it would seem inevitable to be relied upon in critical conditions of the patients, where a rapid diagnosis and prompt treatment would be a visual loss saving measure [35, 36].

Marine sponges are among the most promising sources of new antimicrobial substances [37]. When screening marine sponges for their antimicrobial activity, this revealed the characterization of a wide range of active substances with the isolation of a vast number of antibiotic compounds [38, 39], which are still under experimental investigation. Antibacterial activity substances isolated from marine sponges were characterized as manzamine A and psammaplin A, as reported by Laport *et al*. [37].

Due to the increasing practice of antibiotic abuse, known in developed countries, it has become important to deviate our research from the conventional chemical antibiotics to the natural products that are derived from marine organisms. In a study conducted by Torres *et al.* [38] and Seleghim *et al*. [40] they reported the isolation of substances active against antibiotic resistant bacteria from a hospital. These were the Arenosclerins A-C and haliclonacyclamine E which were isolated from the Brazilian sponge *Arenosclera brasiliensis*.

The most prominent sponges in the Red Sea that grow exposed are *Negombata* sponges [41]. Genus *Negombata* is represented by four species, *N. magnifica, N. corticata, N. kenyensisanda* and a new undescribed

species from Indonesia. The three described species have a brilliant orange-red coloration and a digitate or leafy gross morphology. The two species present in the Red Sea are *N. magnifica* and *N. corticata* [42]. *Negombata* sponge was shown to produce potent cytotoxic macrolides called latrunculins (e.g. latrunculins A and B) in addition to other cytotoxic compounds [43]. Latrunculin B differs from latrunculin A in containing 14 versus 16 membered macrocycles [44]. Experiments performed *in vitro* revealed that latrunculins could inhibit force development in muscles, the microfilament-mediated processes of meiosis [45], fertilization, and early development [46] and even affect protein kinase C signaling [47]. Latrunculin A has been found to disrupt the actin cytoskeleton, leading to deterioration of microfilament bundles, loosening of cell-cell attachment, and cell retraction [48]. These results have raised interest in the potential use of latrunculins as growth inhibitors of some tumor cell lines, and therefore, the possibility for them to serve as prototypes in the discovery and development of novel antitumor agents [49].

Moreover, latrunculins are patented as possible antiglaucoma leads. They were reported to decrease intraocular pressure and increase outflow facility without corneal effects in monkeys [50]. *Negombata magnifica* samples from different locations (Ras Mohamed and Safaga) produced both latrunculins A and B, but in different concentrations. *N. magnifica* collected from Ras Mohamed produced the highest concentration of latrunculin A. Whereas *N. magnifica* collected from Safaga produced the highest concentration of latrunculin B. Moreover, a comparison of latrunculin concentrations in the summer and winter indicated that latrunculin concentrations were generally higher in the winter than in summer [51].

In our study we attempted to investigate the antibacterial activity of crude extracts of marine sponges from the Red Sea in Egypt against two common antibiotic resistant bacteria isolated from postoperative endophthalmitis patients, namely *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In this study, both species of sponge showed antibacterial activity. We revealed that initial screening using aqueous and ethanolic crude extracts of the 2 sponge species showed that they possessed inhibitory activity against *Staphylococcus aureus* more than *Pseudomonas aeruginosa*, where all 6 (43%) *Staphylococcus aureus* culture positive samples were sensitive to the extracts while only 4 (29%) of 8 *Pseudomonas aeruginosa* culture positive samples were sensitive to the extracts. This showed that both sponge species extracts showed more antibacterial activity for gram positive cocci than gram negative bacilli, where *Staphylococcus aureus* is a well known multi resistant bacteria in hospitals leading to nosocomial infections (Hospital acquired infections) e.g. methicillin-resistant *Staphylococcus aureus* (MRSA). Postoperative endophthalmitis would be the end result of this antibiotic resistant pathogen, thus necessitating the need for new natural antibacterial products for treating this serious situation. Although our study is considered an initial screening for antibacterial activity of crude extracts of marine sponge, yet it revealed promising inhibitory effect against *Staphylococcus aureus* isolated from a hospital.

Future investigation for the study of the active fraction of the compound substances and determination of the amount needed to induce bacterial growth inhibition, would be of great value for the treatment of infections due to multi resistant bacteria especially post operatively.

- Polymerase chain reaction detection of microbial DNA in aqueous humor samples may prove a rapid and useful means of diagnosing postoperative endophthalmitis and facilitating management decisions when conventional bacterial culture is negative.

- Polymerase chain reaction based technology was a useful adjunct to conventional culture because when used with aqueous humor samples only, the association of both techniques allowed for a microbiological diagnosis in 76% of cases of postoperative endophthalmitis.

- Red Sea sponges offer a potential for production of novel drugs and prototypes.
- Marine sponges are among the most promising sources of new antimicrobial substances.
- Screenings of marine sponges for antibacterial activity led to the isolation and characterization of a wide range of active compounds, including some promising therapeutic leads. Further studies are needed to know the concentration of the active substance with the aim to use it in the future for treatment of infections caused by multiresistant bacteria.

Table.1 Sequences of primer sets used.

Microorganisms	Primer Sequence	Product Size (bp)	References
Universal primer for bacteria	F OR 27 – 6: GGA GGA AGG TGG GGA TGA C REV 28 – 6: ATG GTG TGA CGG GCG GTGTG	**241 bp**	**Samadi *et al.* [11]**
Staphylococcus aureus	F OR 9-6: CAA TGC CAC AAA CTC G REV 10-6: GCT TCA GCG TAG TCT A	**477 bp**	**Sakai *et al.* [12]**
Staphylococcus epidermidis	GYRB FOR. -9: CAG CAT TAG ACG TTT CAA G GYRB REv. -9: CCA ATA CCC GTA CCA AAT GC	**251 bp**	**Yamada *et al.* [13]**
***Pseudomonas* spp.**	FOR 21-6: GAC GGG TGA GTA AT G CCTA REV 22-6: CAC TGG TGT TCC TTC CTA TA	**618 bp**	**Theodore *et al.* [14]**

FOR= forward REV= reverse GYR= Gyrase bp= base pair

Table.2 Culture and smear results.

Total no. of cases	Direct smear positive (Gram stain) (%)	Culture positive (%)	Both culture and direct smear positive (%)
50	24 (48%) (14 *Ps. aeruginosa* and 10 *S. aureus*)	14 (28%) (8 *Ps. aeruginosa* and 6 *S. aureus*)	14 (28%) (8 *Ps. aeruginosa* and 6 *S. aureus*)

Fig.1 Agarose gel electrophoresis of PCR products using ***Pseudomonas*** genus specific primer. Lane 1: marker. Lane 8: positive control. Lane 9: negative control. Lane 6: negative sample. Positive PCR results are seen in lanes 2, 3, 4, 5 and 7. Product size 618 bp.
+ C = positive control, -C = negative control, M= marker.

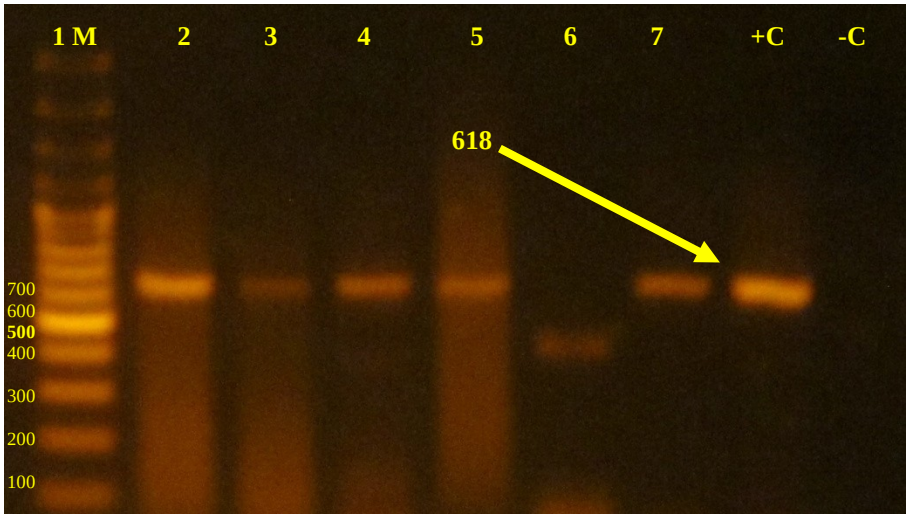
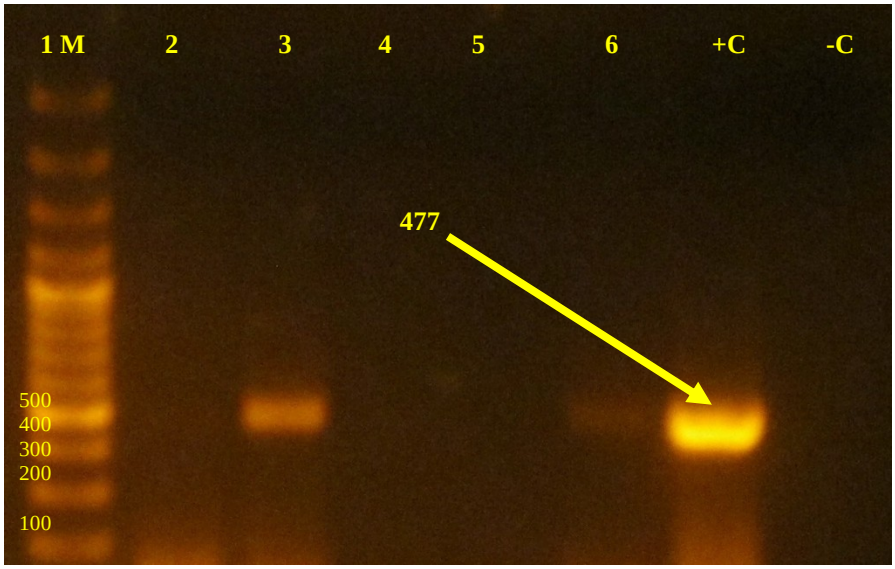


Fig.2 Agarose gel electrophoresis of PCR products using ***Staphylococcus aureus*** genus specific primer. Lane 1: marker. Lane 7: positive control. Lane 8: negative control. Lanes 2, 4, 5 and 6: negative samples. Positive PCR result is seen in lane 3. Product size 477 bp.



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