Activity of Moxifloxacin and Gentamicin Singly and Combined with Diclofenac or Ketorolac against Planktonic and Biofilm Cells of Staphylococci Isolated from Ocular Flora

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ABSTRACT

Ophthalmic surgeons have adopted various measures to reduce the incidence of postoperative endophthalmitis. Most bacteria responsible for postoperative ocular infection are part of the normal microbial flora of the conjunctiva. Therefore, the aim of this study was to evaluate the susceptibility of conjunctival bacterial flora of patients undergoing ocular surgery to the fourth generation fluoroquinolone, moxifloxacin (MOX) and the aminoglycoside, gentamicin (CN) either alone or in combination with diclofenac (VOL) or ketorolac (AC). The most prevalent microorganisms in the ocular flora were coagulase negative staphylococci (CoNS, 62%) and S. aureus (39%). MIC range of MOX was 0.125–4 µg/ml, compared to <0.125-1 µg/ml and 0.125-2 µg/ml in presence of diclofenac (VOL) and ketorolac (AC), respectively. Comparable results were obtained with using CN, though to a lower extent. Moreover, testing the bactericidal effect of MOX and CN, after 30 min and 1h, revealed that MOX is more potent than of CN against all tested strains irrespective to the incubation time, and this effect was more prominent when CoNS MOX-sensitive strains were tested. After 1h treatment, contact time, of the biofilms formed by the tested strains with MOX and CN, the most prominent effect was against CoNS tested which was observed with 500 µg/ml MOX followed by 8 µg/ml CN then 50 µg/ml MOX. However, when the multiresistant, oxacillin-resistant S32 and MRSA13 isolates were tested, MOX had more potent effect than CN, regardless of the concentration used. The results of the present investigation reveal that, VOL obviously potentiates the in vitro antibacterial action of MOX and CN. Moreover, presence of 50 and 500 µg/ml MOX completely prevented the formation of biofilm by the tested strains, but the effect on previously formed biofilm was markedly low. On the other hand, CN was far less effective either against biofilm formation or its eradication.

INTRODUCTION

Endophthalmitis is an uncommon but potentially devastating complication of cataract surgery and often carries a poor prognosis. Consequently, ophthalmologists adopt several measures for its prophylaxis(1,2). Bacteria are the most common group of causative agents of endophthalmitis where Gram positive pathogens are responsible for 60 to 80% of acute infections(3). Previous studies have shown that most bacteria responsible for postoperative ocular infection are part of the normal microbial flora of the conjunctiva and eyelids of the patient(4,5).

Normal microbial flora of the eyelid and conjunctiva has been reported since the 1930s(6-8). The most frequently isolated pathogen is coagulase-negative Staphylococcus (CoNS), followed by Staphylococcus aureus and Streptococcus spp; Gram negative organisms are responsible for 20% of the microorganisms(9,10). Implantation of artificial intraocular lenses into the eye during ophthalmic surgical procedures ensures a nonliving surface on which bacterial pathogens may attach and form more resistant bacteria in biofilms. Despite antibiotic treatment bacteria growing in biofilms might cause inflammation and serious complications(11,12). The various measures adopted to reduce the rate of endophthalmitis vary considerably. The measures currently used by surgeons include instillation of topical antibiotic eye drops before or after surgery, or antibiotics may be added to the irrigation fluid or given as a bolus dose at the end of surgery(1).

Fluoroquinolones are synthetic, broad-spectrum, rapidly bactericidal antibiotics, with good penetration into ocular tissues. Since antibiotic resistance occurred swiftly to the earlier fluoroquinolones (ciprofloxacin or ofloxacin), more potent fluoroquinolones were needed. Fluoroquinolones have activity against normal aerobic flora of the ocular surface, especially Gram positive species which has low
resistance to fourth generation fluoroquinolones. The fourth generation fluoroquinolones have enhanced activity against Gram positive bacteria while retaining potent activity against most of Gram negative bacteria\textsuperscript{(13)}. Topical ophthalmic antibiotic products of fourth generation fluoroquinolones (e.g. moxifloxacin) can deliver antibiotic concentrations directly to the eye that are thousands of times higher than their MICs\textsuperscript{(14)}. Moxifloxacin penetrates better into ocular tissues than gatifloxacin and older fluoroquinolones. Moxifloxacin also has better mutant prevention characteristics than other fluoroquinolones\textsuperscript{(15)}. Gentamicin (CN) is active in vitro against wide range of bacterial isolates including Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus spp., and Gram negative microorganisms. Gentamicin Sulphate Ophthalmic Solution, is 0.3\% Gentamicin Sulphate sterile aqueous buffered solution\textsuperscript{(16,17)}. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used in the treatment of ophthalmic inflammatory disease, and for the treatment of itching associated with seasonal allergies, e.g. hay fever\textsuperscript{(18)}. In addition, they can be used prior to cataract surgery to prevent the pupil constricting during the surgery\textsuperscript{(19)}. Currently four topical NSAIDs eye drops are available among which are: diclofenac (topical diclofenac sodium 0.1\% ophthalmic solution) and Ketorolac (topical ketorolac tromethamine 0.5\% ophthalmic solution). A previous report suggested that, the use of wide-spectrum antibiotic associated with anti-inflammatory eye drops has a significant effect on the reduction of a potential infectious and inflammatory process after refractive surgery\textsuperscript{(20)}. In contrast other work group reported that, NSAIDs did not alter the efficacy of the antibiotic however it decreased the aqueous humour concentration of the tested antibiotics\textsuperscript{(21)}. Consequently, the main objectives of the present work is to study: (i) the susceptibility of the conjunctival bacterial flora to commonly used antibiotics; (ii) the effect of NSAIDs on the MICs of MOX and CN ophthalmic solutions; (iii) the bactericidal activity of the in-use concentrations of the tested antibiotics against some selected isolates and (iv) the efficiency of the in-use concentrations of the tested antibiotics to prevent biofilm formation or to eradicate the pre-existing one.

**MATERIALS & METHODS**

**Bacterial Strains and Susceptibility Testing.**

The tested clinical isolates in this work were obtained through the courtesy of the Department of Microbiology, Faculty of Medicine, Alexandria University Hospital. The specimens from 80 patients undergoing intraocular surgery taken from the routine evaluation of the conjunctival bacterial flora were cultured on 5\% blood agar, Mannitol salt agar and MacConkey’s agar. In addition, catalase and coagulase tests were carried out to identify the staphylococcal isolates\textsuperscript{(22)}. The susceptibility of staphylococcal isolates to 13 antibiotics, was determined by disc-diffusion technique using Müller-Hinton agar (Oxoid, England). The antibiotic discs were purchased from Oxoid Ltd. England. The resultant inhibition zones were recorded and interpreted as sensitive, intermediate or resistance according to CLSI 2003 guidelines\textsuperscript{(23)}. Moxifloxacin discs 1 \(\mu\)g, suggested for testing most of the organisms except pseudomonads, were prepared using blank discs as previously reported\textsuperscript{(24)}. A zone diameter breakpoint of \(\geq 20\) mm denoted sensitivity of Enterobacteriaceae and staphylococci\textsuperscript{(24)}.

To be certain of resistance of the selected strains to MOX and CN, the breakpoint concentration\textsuperscript{(23,24)} of each antibiotic was prepared by agar dilution in Müller-Hinton agar plates (2 \(\mu\)g/ml for MOX and 8 \(\mu\)g/ml for CN) and aliquotes of the appropriate dilution of the tested strains were dropped on the plates.

**Antimicrobial Agents and NSAIDs.** Moxifloxacin ophthalmic solution 0.5\% (Vigamox\textsuperscript{®}; Alcon Laboratories, Inc., Fort Worth, TX, USA). Gentamicin sulphate (Schering Plough Corp), Diclofenac sodium (Novartis New Zealand Limited) ketorolac tromethamine 15mg/ml Ketolac\textsuperscript{®} ampoules were used in place of Acular\textsuperscript{®} eye drops.

**Determination of MICs.** The minimum inhibitory concentrations (MICs) of MOX and CN were determined either alone or in presence of 200 \(\mu\)g/ml diclofenac (VOL) or 1000 \(\mu\)g/ml ketorolac (AC) against 10 selected staphylococcus strains using the macrobroth dilution technique outlined by CLSI 2003\textsuperscript{(23)}. The concentrations of VOL and AC used were a 5 fold dilution of the eye drops (Voltaren\textsuperscript{®} and Acular\textsuperscript{®}, respectively) concentration. The MICs were determined in triplicates in Müller-Hinton broth. An overnight broth suspension was first compared with 0.5 ‘McFraland Opacity
Standard’ which was equivalent to approximately $10^8$ CFU/ml and properly diluted to a final bacterial concentration of $10^5-10^6$ CFU/ml was used. After incubation at 35°C for 18-22 h the tubes were examined for macroscopic growth. The lowest antibiotic concentration, or antibiotic/NSAID combination, causing complete inhibition of growth represented the MIC.

**Determination of Bactericidal Activity.** Bactericidal activity was studied using antimicrobial agents at the following concentrations: MOX 500 µg/ml, MOX 50 µg/ml and CN 8 µg/ml. The concentration for each agent was chosen carefully to reflect the bolus dose of MOX injections and the CN infusion during cataract surgery (25, 26). For *in vitro* studies, these represented the most clinically relevant concentrations. The bactericidal activity of antibiotics was tested against susceptible strain, resistant strains and MRSA strain. Time–kill studies were performed in sterile saline at 37°C with a starting inoculum of $5 \times 10^5$ CFU/ml. Proper controls were included. Aliquots were removed, after 30 min and after 1 h, serially diluted in sterile saline filtered through 0.45 µm membrane filter and washed by passing 10 ml sterile saline to overcome the antibiotic carryover effect. The number of survivors on each membrane filter was determined by vortexing the filter with saline which was decimally diluted and used for viable count (27).

**Biofilm Formation.** Determination of the ability of selected staphylococci strains to form mature biofilm in the presence of the previously mentioned concentrations of MOX and CN was tested as reported by Borriello *et al* in 2006 (28). Also the ability of MOX and CN to eradicate pre-formed biofilm has been investigated where the formed mature biofilms were subjected to the selected antibiotic concentrations or saline (control) for 1h followed by sonication for 5 min, serially diluted in sterile saline and plated onto Müller-Hinton agar plates to quantify CFU/ml.

**RESULTS & DISCUSSION**

Results in previous studies characterizing endophthalmitis isolates demonstrate that Gram positive microorganisms account for 90% or more of pathogens isolated in culture positive cases of postcataract surgery endophthalmitis with coagulase-negative staphylococci (*Staphylococcus epidermidis*) and *Staphylococcus aureus* representing the leading causes (29-32).

Among the prophylactic measures for reducing the actual rate of endophthalmitis and reducing the bacterial load after surgery, is using topical antibiotics drops pre- and postoperatively, injecting a bolus dose of MOX (up to 500 µg/ml) or CN infusion (8 µg/ml) during the cataract surgery. Previous studies have shown that, most bacteria responsible for postoperative ocular infection are part of the normal microbial flora of the conjunctiva and eyelids of the patient (4,5).

Consequently, the purposes of this study were to determine the normal ocular bacterial flora isolated from 80 patients undergoing ocular surgery and to evaluate their *in vitro* susceptibility of the Gram positive isolates to MOX and CN alone in presence of NSAIDs. Not only the susceptibility of the planktonic bacterial cells was evaluated, but also that of the cells in biofilm.

Seventy four percent of the specimens from 80 patients undergoing intraocular surgery taken from the routine evaluation of the conjunctival bacterial flora showed positive cultures. Staphylococci were found in 92% (55 isolate), which was the highest prevalence genus-wise. Most of the bacteria identified by culture were coagulase negative staphylococci "CoNS" (34 isolate, 62%), while coagulase-positive *Staphylococcus aureus* (CoPS) was isolated in 39% (21 isolate) of all swabs and 11% for the Gram negative rod. Only 2 MRSA strains were isolated. These results were coherent with the previously reported data (33, 34).
Antibiotic susceptibility of the 55 isolated Gram positive staphylococcal strains to 13 antibiotics was determined by the disc-diffusion method. The percentage of the resistance of the tested isolates ranged from 0% for vancomycin to 81% for penicillin, Fig. 1. Thirty four percent of the tested strains were resistant to oxacillin and penicillin indicating resistance to all currently available β-lactam antibiotics (CLSP 2003)\(^{23}\). The tested isolates were found to be equally resistant to the fluoroquinolones; levofloxacin and lomefloxacin, however, lower level of resistance was recorded in case of MOX (9.5%), Fig. 1. Similarly, higher rates of resistance to ciprofloxacin or levofloxacin have been reported in some areas of Canada and in Hong Kong\(^{35,36}\).

Prior to cataract surgery, broad spectrum antibiotics are used to reduce the normal bacterial flora while NSAIDs are used to prevent the pupil constricting during the surgery. Postoperatively, the antibiotic is continued to reduce the risk of ocular infections and the NSAIDs are used to reduce the pain and inflammation due to the surgery\(^{28}\). For this reason, it was essential to investigate the effect of NSAIDs on the antibacterial activity of MOX and CN.

The MICs of MOX and CN either alone or in presence of diclofenac (VOL) or ketorolac (AC) against 10 selected staphylococcus strains (2 MRSA, 3 S. aureus and 5 CoNS) were determined using the macrobroth dilution technique. Breakpoints for susceptibility to MOX have been approved by FDA and are listed in the manufacturer's package insert\(^{37}\). The breakpoints from the CLSI have not yet been reported. According to the package insert, staphylococcus species and the Enterobacteriaceae are susceptible at an MIC of 2.0 µg/ml, intermediately resistant at 4.0 µg/ml, and resistant at 8.0 µg/ml\(^{37}\), while high and low breakpoints of ≥8 mg/L and ≤4 mg/L, respectively, are suggested for CN by the CLSI 2003\(^{23}\). Table I shows that, all but two of the tested strains were sensitive to MOX (1 MRSA and 1 CoNS) which were intermediately resistant to MOX. On the other hand, 8 out of the tested 10 isolates were (80%) CN-resistant. The 2 sensitive isolates were CoNS. Thus resistance to CN is much more common than resistance to MOX.

In a study in Spain on 245 clinical strains of methicillin-resistant staphylococci including 225 MRSA and 20 methicillin-resistant CoNS, a median MIC of MOX and CN of 4 µg/ml and 32 µg/ml, respectively for the tested MRSA strains and of 1 µg/ml and 128 µg/ml, respectively for the tested CoNS was obtained. All but one of the CoNS isolates tested, were CN-resistant\(^{38}\). On the other hand, Kotlus et al reported that MRSA ocular isolates exhibited a relatively high rate of in vitro resistance to all fluoroquinolones tested, including the fourth generation and added that the isolates were
found to be highly sensitive to vancomycin and gentamicin (39). The MICs of both antibiotics is dramatically affected by the presence of Voltaren®, while the Acular® had little or no effect on the MICs of the tested antibiotics (Table I). Fifty percent of the strains that were resistant to CN, when tested alone, became sensitive to CN in the presence of VOL, where the MICs were reduced by at least 3 or 4 folds. However, in the presence of AC the MICs of 60% of the tested strains were unchanged and the other 40% showed 1 or 2 fold decrease in their MICs. For MOX, MICs of 60% of the tested strains were reduced to less than the minimum tested concentration, while only 2 strains showed one fold reduction in their MIC when AC was tested (Table I).

A previous work reported that, diclofenac-gentamicin (DR1352/1) eye drops were more effective than gentamicin eye drops and appeared to be as safe in the control of post-cataract surgery inflammation (40). In vivo study suggested that, the use of wide-spectrum antibiotic associated with anti-inflammatory eye drops had a significant effect on the reduction of a potential infectious and inflammatory process after refractive surgery (20).

Table I: Antibacterial activity of MOX and CN in absence and presence of VOL and AC

<table>
<thead>
<tr>
<th>Isolate code (coagulase reaction)</th>
<th>MIC in µg/ml</th>
<th>MOX</th>
<th>MOX + VOL</th>
<th>MOX + AC</th>
<th>CN</th>
<th>CN + VOL</th>
<th>CN + AC</th>
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<tbody>
<tr>
<td>MRSA 1</td>
<td>0.125</td>
<td>&gt;0.125</td>
<td>0.125</td>
<td>16</td>
<td>&lt;1</td>
<td>4</td>
<td></td>
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<tr>
<td>MRSA 13</td>
<td>4</td>
<td>0.125</td>
<td>2</td>
<td>&gt;128</td>
<td>16</td>
<td>&gt;128</td>
<td></td>
</tr>
<tr>
<td>S 32 (CoPS)</td>
<td>2</td>
<td>&lt;0.125</td>
<td>2</td>
<td>&gt;128</td>
<td>32</td>
<td>&gt;128</td>
<td></td>
</tr>
<tr>
<td>S 37 (CoPS)</td>
<td>1</td>
<td>&lt;0.125</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>S 39 (CoPS)</td>
<td>0.5</td>
<td>&lt;0.125</td>
<td>0.5</td>
<td>8</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>S 29 (CoNS)</td>
<td>1</td>
<td>&lt;0.125</td>
<td>1</td>
<td>4</td>
<td>&lt;1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>S 30 (CoNS)</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
<td>&gt;128</td>
<td>8</td>
<td>&gt;128</td>
<td></td>
</tr>
<tr>
<td>S 31 (CoNS)</td>
<td>0.5</td>
<td>&lt;0.125</td>
<td>0.5</td>
<td>2</td>
<td>&lt;1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>S 34 (CoNS)</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>&gt;128</td>
<td>16</td>
<td>&gt;128</td>
<td></td>
</tr>
<tr>
<td>S 51 (CoNS)</td>
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<td>0.25</td>
<td>1</td>
<td>16</td>
<td>4</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

(CoPS); Coagulase positive staphylococci, (CoNS); Coagulase negative staphylococci

Bactericidal activity was studied using MOX 500 µg/ml, and 50 µg/ml and CN 8 µg/ml. These concentrations were chosen carefully to reflect the bolus dose of MOX injections and the CN infusion during cataract surgery (25,26). For in vitro studies, these represented the most clinically relevant concentrations. Bactericidal efficacy of antibiotics were tested against susceptible, resistant and MRSA strains. Figures 2 and 3 show that the bactericidal activity of both concentrations of MOX is more potent than that of CN against all tested strains irrespective to the incubation time. The bactericidal activity of MOX could be better observed against the MOX-sensitive CoNS S29 and S30 isolates than on the MOX-resistant (S. aureus S32 and CoNS S34) and MRSA strains. The least bactericidal action of MOX and CN was observed against MRSA strain. In a previous report, MOX at 50 µg/ml against some ocular staphylococcal isolates demonstrated significant killing (>99.5%) in 60 min (41). Another report noted more rapid speed of kill with MOX compared with other nonfluoroquinolone topical ocular antibiotics (tobramycin, gentamicin, polymyxin B/trimethoprim, or azithromycin) against Staphylococcus aureus, Streptococcus pneumoniae, and Haemophilus influenzae infections. Moxifloxacin achieved a 99.9% killing after 1 h contact time for S. aureus (42).
Implantation of artificial intraocular lenses into the eye during ophthalmic surgical procedures ensures a nonliving surface on which bacterial pathogens may attach and form biofilms. Despite antibiotic treatment, bacteria growing in biofilms might cause inflammation and serious complications \(^{(43)}\).

The ability of selected staphylococcus strains to form mature biofilm in the presence of the previously mentioned concentrations of MOX and CN and the ability of the same concentrations of MOX and CN to eradicate pre-formed biofilm has been investigated. All the tested isolates, even the MRSA isolate, were unable to form biofilm in the presence of the tested MOX concentrations (data not shown). On the other hand, Fig. [4] shows that CN had little or no effect on the biofilm formation by the tested CoNS (S34, S30) isolates. However, the most potent effect was that observed when testing MRSA13 isolate as shown in Fig. [4].

Gaál et al reported that, ciprofloxacin administration was able to reduce significantly the number of attached cells (\textit{S. aureus} and CoNS strains) on the surface of acrylic lenses, where the effect was concentration dependant. They also reported that, tobramycin treatment was able to inhibit significantly the attachment of \textit{S. aureus} cells \(^{(49)}\).
Fig. 4: Biofilm formation in the presence of 8 µg/ml CN.

After 1h treatment of the pre-formed biofilms of the tested strains by MOX and CN, the most prominent effect was observed against CoNS (S30 and S34) at 500 µg/ml MOX followed by 8 µg/ml CN then 50 µg/ml MOX Fig. [5]. However, when the multiresistant, oxacillin-resistant S32 and MRSA13 isolates were tested, MOX had more potent effect than CN, regardless of the concentration used Fig. [5].

Fig. 5: Eradication of established biofilms by selected concentrations of MOX and CN

Despite the debate on antibiotic prophylaxis we presented in our experiments that a single antibiotic administration can decrease the attachment of bacterial cells to the surface of acrylic intraocular lenses, and might be effective in the prevention of postoperative endophthalmitis, that is a rare but serious complication of ophthalmic surgery. 

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دراسة نشاط الموكسيفلوكساسين والجيبتاميسين كلا على حدة وفي وجود الديكوفيناك أو الكيتوكلوروكال ضد خلايا العوائل والغشاء الحيوي للبكتيريا العنقودية المعزولة من الفنورا البصرية

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لقد اتخذ جراحى العيون مختلف التدابير من أجل خفض حالات الأنوفاليميتس في العين ما بعد الجراحة. وقد لوحظ أن معظم البكترىا المسببة لداء العيون بعد الجراحه هي بعض من البكترىا البكتيرية الطبيعية للمستقبلة. ولذلك، فإن الهدف من هذه الدراسة هو تقييم حساسية البكترىا البكتيرية الطبيعية للمستقبلة المعزولة من الأشخاص الذين يعانون من الجراحه البصرية إلى الجيل الرابع من موكسيفلوكساسين (MOX) والحمض الأمينوكليكسيدين (CN). وقد أوضح النتائج أن أكثر الكائنات انتشاراً في الفنورا الطبيعية للعين الميكروفينوكس السالبة لإنزيم الكوأجولاز (CoNS) (26% MOX) والبكتيريا الذهبية المعزولة (29%)، وقد كانت أقل التركيزات المثبتة لنمو البكتيريا لم يتجاوز من 0.125 - 0.25 ميكروجرام/مل بالمقارنة بينها والليكولاور (VOL) والليكولاور (AC) للميكروفينوكس السالبة لإنزيم الكوأجولاز (MOX).

ندرة من 0.25 - 1 ميكروجرام/مل و 2 ميكروجرام/مل على التوالي، ولقد كانت النتائج مماثلة مع CN والعكس صحيح، حيث اتضحたら النتائج MOX و CN على القائمة بعد نصف ساعة وتلك بعد ساعه. وقد أوضح النتائج CN أن فاعلية البكترىا البكتيرية المحمولة بعض النظير عن فترة الحضارة، وقد كان هذا التأثير أكثر MOX في البكترىا البكتيرية المحمولة بعض النظير عن فترة الحضارة، وقد كان هذا التأثير أكثر MOX في البكترىا البكتيرية المحمولة بعض النظير عن فترة الحضارة. وبناءً على النتائج، فإن هذا التأثير أكثر وضوحاً مع CN، مثلاً في البكترىا البكتيرية المحمولة بعض النظير عن فترة الحضارة.

لكن عندما تم اختبار عزلات متعددة المقاومة ومعاقبة ميكروجرام/مل CN، فإنها كانت أقل فاعلية ضد CN من CN، بينما تم اختبار CN، كما كان هذا التأثير أكثر وضوحاً مع CN. ولذلك فإن CN، مثلاً في البكترىا البكتيرية المحمولة بعض النظير عن فترة الحضارة.

ويبدو أن هذه النتائج تدعم فعالية CN في أخرى من CN، وقد وجد أن CN من ميكروجرام/مل من CN، مثلاً في البكترىا البكتيرية المحمولة بعض النظير عن فترة الحضارة. وبناءً على النتائج، فإن فعالية CN في أخرى من CN، وقد وجد أن CN من ميكروجرام/مل من CN، مثلاً في البكترىا البكتيرية المحمولة بعض النظير عن فترة الحضارة.