The Potency of a Combined Diclofenac-Ampiclox against *Streptococcus Pyogen* Isolated from Patients with Pharyngitis: A pilot study

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Abstract

Background: Pharyngitis, scarlet fever, and rheumatic fever are of the major infectious problems caused by *Streptococcus pyogen*. Emergence of antibiotic-resistant strains makes such infection, its rheumatic valvular disease, and glomerulonephritis complications a serious clinical challenge especially in children.

Aims and objectives: In a maneuver of overcoming *S. pyogen* resistance and improve its response to antibiotic, a non-steroidal anti-inflammatory drug NSAID is repurposed as antimicrobial against *S. pyogen*. In intention to be combined with the classical antibiotic recommended by WHO guideline of care. The combination was assessed for its MIC and combination index against *S. pyogen*.

Materials and Methods: A multidrug-resistant strain of *S. pyogen* was isolated from patients, suffering pharyngitis. The most serious isolate was cultivated for MIC determination of ampiclox-diclofenac combination in comparison with each alone to determine the combination index.

Results and conclusions: There was a significant synergism between ampiclox-diclofenac (index < 1) at P= 0.012 and Z score= 2.3 further confirmation of MIC folds of dilutions are to be assessed to obtain reasonable evidence.

Recommendations: We recommended to further confirm the antistreptococcal effect of the combined ampiclox-diclofenac on more dilutions of MIC assay and to analyze data of this combination in patients with *S. pyogen* pharyngitis.

Keywords: Multidrug resistance, *S. pyogen*, diclofenac, ampiclox, combination index.

Introduction

*Streptococcus pyogenes* is a species of gram-positive bacteria in the genus Streptococcus. Sore throat is a frequent presenting complaint about outpatient medical visits, and infection with *S.* pyogenes is diagnosed in 20 to 40% of pharyngitis cases in children and in 5 to 15% in adults (1,2). *Streptococcal pharyngitis* ("strep throat") resembles viral pharyngitis. Of children with a sore throat, 15% to 36% have group A beta-hemolytic *Streptococcus* (GABHS). The rate is less than 20% for adults. Ten percent to 25% of general, asymptomatic population are carriers for group A *Streptococcus* (3,4).

Organization A *Streptococcus* pharyngitis may be mild to moderate or associated with excessive fever, anterior cervical lymphadenopathy, tonsillar exudates and raised peripheral white cells. signs and symptoms normally clear up after three-five days, suppurative headaches mainly in peritonsillar and retropharyngeal areas, lymphadenitis, otitis media, mastoiditis, and meningitis. Non-suppurative forms include scarlet fever and acute rheumatic fever or acute streptococcal glomerulonephritis. whilst most instances are benign, scarlet fever changed into often deadly without antibiotic treatment. Many cases possibly represented what might be regarded as streptococcal toxic surprise syndrome STSS (5,6,7).

Laboratory findings include a positive throat culture for β-hemolytic streptococci and a total white blood cell count usually exceeding 12,000/mm3, with increased numbers of PMNs. The test for C-reactive protein is usually positive (8).

The antimicrobials used against *Streptococci pyogen*

Untreated streptococcal pharyngitis normally resolves few days. After onset antibiotics medications shorten the duration of the extreme illness through about sixteen hours. The primary purpose of treatment with antibiotics is to reduce the threat of headaches inclusive of rheumatic fever and retropharyngeal abscesses (9,10). Antibiotics prevent acute rheumatic fever if given during nine days of the onset of signs (11,12). Antibiotic regimens recommended for *Streptococcal Pharyngitis*. Penicillin V orGis the first line treatment while Cephalexin, Azithromycin or Clarithromycin is given to patients allergic or resistant to penicillin (13).

An antimicrobial activity like of the NSAID against other bacteria

The antimicrobial activity of NSAID was repeatedly assessed against different types of bacteria. Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) used to treat pain and inflammatory disorders, and dysmenorrhea (14,15). Mechanism of diclofenac action. The number one mechanism responsible for its anti-inflammatory, antipyretic, and analgesic motion is by inhibiting of prostaglandin synthesis through inhibition of the transiently expressed prostaglandin-endoperoxide synthase-2(PGES-2) also called cyclooxygenase-2(COX-2). It additionally reveals the bacteriostatic effect by inhibiting bacterial DNA synthesis (16,17).

Over the years, many researchers have attempted to modify diclofenac moiety to synthesize potential and therapeutic agents with anti-bacterial, anti-tubercular, and anti-tumor activity (18,19). Recently, Bhandari et al., synthesized and studied the pharmacological activities (anti-inflammatory, analgesic, and ulcerogenesis) of novel S-substituted phenacyl-1, 3, 4-oxadiazole-2-thione and Schiff bases of diclofenac (20). In contemporary, Palkar et al., have reported the synthesis, pharmacological screening and in silico studies of a new class of diclofenac analogs as promising anti-inflammatory agents (21). 1, 3, 4-thiadiazole is an imperative scaffold since several of these derivatives are known to be associated with multiple biological activities such as anti-allergic, antibacterial, anti-cancer, anti-tubercular, and immunosuppressive (22).
Study objectives

1- To isolate a multidrug resistant strain of S. pyogen from clinical cases of pharyngitis.

2- To determine the MIC value of diclofenac and ampiclox against S. pyogen.

3- To calculate the combination and interaction index between the ampiclox and diclofenac to determine the combination index.

Materials and Methods

The study design was an in vitro experimental model for assessing the MIC for different single and combined antimicrobials.

Samples election

Streptococcus pyogen was isolated from a pharyngeal swab of 10 patients. Ages of the patients ranged from 7 to 69 years of both sexes (6 females and 4 males). All those patients complained from S. pyogen pharyngitis confirmed by pharyngeal swab and culture. The isolate was selected to be of higher virulence of MIC assessment. Virulence factor was determined on the base of clinical severity, serotyping and antimicrobial resistance. In this study, we considered the high resistant strain of S. pyogenas it revealed MDR criteria on disc diffusion assay. The patients enrolled in the study were randomized on the base of any entry case for pharyngeal swab culture and sensitivity referred to AL-Rawan private Lab in Najaf who showed positive S. pyogen culture. Excluded cases were those with immunocompromise status, patients taking antibiotics within 3 days prior to swab cultures, extreme ages, culture negative cases, susceptible S. pyogen strain or least resistance for single antibiotics, pharyngitis due to other bacterial infections like staph aureus pharyngitis. In addition to that, patients with coadministered drugs for chronic illnesses were selected on the base that polypharmacy is not a bias on S. pyogen growth like metoprolol and tolbutamide.

The Ethical form was fulfilled. The patients were indirect cases from medical care referral for pharyngeal swab culture. No one was requested for swab culture that is not needed as a part of mandatory care.

The Antimicrobial MIC Assay:

A stalk solution of the following ingredients was prepared:

Diclofenac alone 100µg / ml, ampiclox alone of 100µg / ml and 100µg / ml of the combined two agents were prepared. Two-fold dilutions were used. S. pyogen was distributed homogeneously on all wells to be ready for 2×serial dilutions of test drugs.
Figure (1): A Diagram represents the main steps of NSAID, repurposing study against *S. pyogen* in combination with common guideline antibiotics.

Table (1): The main materials used in MIC determinations, their vendor, source company and dosage form.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Source contain</th>
<th>Source company</th>
<th>Dose</th>
<th>Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diclofenac</td>
<td>Cipla</td>
<td>India</td>
<td>1 g</td>
<td>Powder form</td>
</tr>
<tr>
<td>Ampiclox</td>
<td>Cipla</td>
<td>India</td>
<td>1 g</td>
<td>Powder form</td>
</tr>
<tr>
<td>Nutrient Broth medium</td>
<td>Hanover</td>
<td>Germany</td>
<td>50g</td>
<td>Powder form</td>
</tr>
<tr>
<td>NaCl</td>
<td>Pioneer</td>
<td>Jordan</td>
<td>1 pint</td>
<td>Infusion fluid</td>
</tr>
<tr>
<td>DDW</td>
<td>local</td>
<td>Iraq</td>
<td>1 liter</td>
<td>Liquid</td>
</tr>
</tbody>
</table>

Note: DDW = deionized distilled water

Preparation of the stalk test solution

A Standard drug weight equivalence to 100 µg was dissolved in 1 ml of DDW to produce a concentration of 100 µg/ml for further serial 2 x dilutions within the MIC assay.

Count of the *S. pyogen* CFU was scored as 0 = no growth, 1 = mild growth 10^6 CFU, 2 = moderate 10^7 CFU, 3 = heavy growth >10^9 CFU. This score was estimated as the outcome corresponds each serial dilution.
Results:

Table (2): In vitro susceptibility assay by disc-diffusion method for S. pyogen against different standard antimicrobials.

<table>
<thead>
<tr>
<th>Type of Antibiotic</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalothin (KF)</td>
<td>R</td>
</tr>
<tr>
<td>Ceftriaxone (CRO)</td>
<td>H.S</td>
</tr>
<tr>
<td>Cefepime (FEP)</td>
<td>M</td>
</tr>
<tr>
<td>Doxycycline (DO)</td>
<td>H.S</td>
</tr>
<tr>
<td>Tobramycin (TB)</td>
<td>M</td>
</tr>
<tr>
<td>Ciprofloxacin (CIP)</td>
<td>R</td>
</tr>
<tr>
<td>Meropenem (MEM)</td>
<td>H.S</td>
</tr>
<tr>
<td>Nitrofurantoin (F)</td>
<td>H.S</td>
</tr>
<tr>
<td>Amoxicillin (AX)</td>
<td>H.S</td>
</tr>
<tr>
<td>Levofoxacin (LEV)</td>
<td>H.S</td>
</tr>
<tr>
<td>Trimethoprim (TMP)</td>
<td>H.S</td>
</tr>
<tr>
<td>Azithromycin (AZM)</td>
<td>H.S</td>
</tr>
<tr>
<td>Amikacin (AK)</td>
<td>H.S</td>
</tr>
<tr>
<td>Vancomycin (VA)</td>
<td>H.S</td>
</tr>
</tbody>
</table>

Note: R=Resistant, M= Moderate sensitive, H. S= Highly Sensitive.

A confirmation disc diffusion assay for S. pyogen susceptibility was done in order to profile the strain of the isolate. In this study we intentionally selected the most multidrug resistant strain to be subjected for MIC assay.

Table (3): The MIC50 values of diclofenac and ampiclox against S. pyogen culture.

<table>
<thead>
<tr>
<th>Conc. µg / ml</th>
<th>Diclofenac</th>
<th>Ampiclox</th>
<th>Diclofenac Ampiclox</th>
<th>P Mann-Whitney test</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0.012</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6.25</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

The comparison was between the MIC of growth score of each drug alone in relation to the combined group in order to determine type of interaction. P value was 0.012 and Z-score was 2.50. The sample size was less than 10, so that the assessment needs for more serial dilutions. However, there was a significant synergistic effect between diclofenac and ampiclox although ampiclox showed resistance in MIC assay. The score of culture growth was designated as 0 = no growth, 1 = mild growth 10^6 CFU, 2 = moderate 10^7 CFU, 3 = heavy growth >10^9 CFU.
The anti-streptococcus interaction index

Table (4): The interaction index of the combined ampiclox-diclofenac against *S. pyogens*.

<table>
<thead>
<tr>
<th>Combination</th>
<th>Interaction index (combination index)</th>
</tr>
</thead>
<tbody>
<tr>
<td>diclofenac-ampiclox</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

The score of the final dilution was taken for determining the CI. CI of diclofenac-ampiclox was less than 1, which indicates a synergetic effect provided that MIC of diclofenac is more than that of diclofenac-ampiclox combinations.

Discussion:

*S. pyogen* revealed many isolates in the collected throat swab samples that were resistant to common beta-lactam antibiotics. Moreover, some other antimicrobials became ineffective in treating many isolates of *S. pyogens* like aminoglycosides and sulfonamides. In concern of the isolate used in this study, *S. pyogens* showed resistance to both cephalothin and ciprofloxacin. (Table 2).

Although this strain of *S. pyogen* showed high susceptibility to amoxicillin in the standard disc diffusion assay, however, this strain revealed a high resistance to ampiclox even at a concentration of 100 µg /ml in MIC assay. On the other hand, *S. pyogen* may cause fatal sequelae in children like rheumatic valvular disease. Methods of mitigation of *S. pyogen* resistance are then urgent. Many NSAIDs like diclofenac, showed some antimicrobial effects against variable strains of microorganisms. Combining NSAIDs with the common guideline antibiotics for treating *S. pyogen* pharyngitis is recommended medical care in this type of infection. Diclofenac showed structural similarity with some quinolones. Combining diclofenac with the extended spectrum antibiotic, ampiclox is a plausible rationale for this medical care in an attempt to overcome resistance. The combination revealed a significant anti-streptococcal effect. However, diclofenac alone was as effective as the combined formula. A study by Altman *et al* showed diclofenac have led to the creation of a broad array of drug products designed to treat multiple inflammatory and painful conditions.

Although synergism was significant between diclofenac and ampiclox, diclofenac alone showed the same inhibitory effect with interaction index < 1 (table 4). However, in real culture diclofenac showed a minute difference lower than that of the combination that was not expressed in the digital score of growth, so further bacterial count may reveal the difference in MIC between diclofenac and the combination. Moreover, further folds of dilution are to be conducted in further studies to determine the exact MIC of the test drugs in micro molar scale.

Conclusion:

There was a significance synergistic effect obtained from combining ampiclox with diclofenac against *S. pyogen* isolate in culture although this isolate was resistant to ampiclox alone. However, this synergism was not significant when diclofenac inhibitory effect test is considered.

Recommendations:

We recommended to further confirm the antistreptococcal effect of the combined ampiclox-diclofenac on more dilutions of MIC assay and to analyze data of this combination in patients with *S. pyogen* pharyngitis.
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8- Amy E. Bryant, Dennis L. Stevens, in Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition), 2015.


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