Synergistic Influence of Tetracycline on the Antibacterial Activities of Amoxicillin Against Resistant Bacteria

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ABSTRACT

In this study, the in vitro influence of antibacterial activity of amoxicillin and tetracycline on each other was investigated by macrobroth dilution and checkerboard assay methods. Each of the antibiotics as well as their combinations proved inhibitory against all the tested organisms with the exception of Acinetobacter calcoaceticus showing high level of resistance. The inhibition zones produced by the combination of tetracycline and amoxicillin with those obtained from each antibiotic showed synergistic interaction which is concentration dependent. The checkerboard assay results showed 87.5% synergy and 12.5% antagonism with a high reduction in the Minimum Inhibitory Concentrations (MICs) and a strong bactericidal activity against the test organisms. Results of macrobroth dilution and checkerboard assays are complementary and indicated a strong synergistic interaction between amoxicillin and tetracycline.

Key words: Antibiotics, checkerboard assay, macrobroth dilution, synergy, antagonism

INTRODUCTION

The changing antimicrobial sensitivity pattern of causative organisms has posed a therapeutic challenge in treating patients in the recent time. Unregulated provision of antibiotics, dispensing of insufficient doses, reduced adherence to complete dose regimens and the poor quality of the drug supply (Goossens et al., 2005; Blomberg et al., 2007; Cars et al., 2008), decreasing effectiveness of conventional antimicrobial drugs (Park et al., 2011) coupled with the overuse or misuse of antibiotics accelerating antibiotic resistance (Collignon et al., 2009; Love et al., 2011) and Extended Spectrum Beta Lactamases (ESBL) (Gururajan et al., 2011) has caused serious problems due to the rapid emergence of multidrug-resistant pathogens as well as creating a major challenge for the public health in the world (Zhang et al., 2011). Resulting from these challenges, antibiotic resistance threatens human health worldwide (Karou et al., 2009; Hogberg et al., 2010), affect treatment decisions, patient outcome, health care expenditure and public perceptions of health care delivery (Okeke et al., 2005; Sosa et al., 2010). Since there is a growing awareness of public health concerns associated with the emergence of drug-resistant strains of bacteria (Raghunath, 2008) and antibiotic resistance is currently involving most of the known classes of natural and synthetic antibiotics (Pednekar et al., 2011), there is an urgent need for antibiotics with novel scaffolds and mechanism of actions. In response to identifying alternative means to combat these situations, combination therapy or polypharmacy has been acknowledged.
Combination therapy or polypharmacy (Alam et al., 2011) which is the concurrent use of two or more medications in a single patient has increased progressively with increasing global population, progression of the existing infections, onset of new microbial infections and development of resistance in infectious agents. It is known to be superior to single modality therapy (Masoudzadeh and Khalilian, 2007), able to minimize side effects (Haidari et al., 2008) and may be more effective in the treatment of resistant infections. The mitigating factors may include proliferation of new antimicrobial agents thought to be better tolerated than previously available drugs, the increasing tendency to treat partial response by introducing additional agents and increased use of drugs to alleviate adverse effects accompanying use of existing anti-infective agents. However good it is by simplifying treatment and improve drug compliance by reducing the burden of taking multiple drugs (Chobanian et al., 2003; Mancia et al., 2007), the mechanistic basis of many drug-drug interactions is well established (Stockley, 2002). The drug-drug interactions can occur as a result of changes in pharmacodynamics and/or pharmacokinetics. A pharmacodynamic interaction occurs when drugs act on the same receptor, site of action, or physiologic system. A drug or substance that interferes with or accentuates the absorption, distribution, or elimination of a second drug produces a pharmacokinetic interaction. While some drug interactions are beneficial, others are clinically harmful and may eventually result in therapeutic failures.

To avoid unpleasant pharmaco logical and therapeutic actions related to drug-drug interactions (Rashidul Barri et al., 2000) and forestall therapeutic failures, development of bacterial resistance and increased cost of treatment often associated with the inept polypharmaceutical practices, researches in synergistic mechanisms of action for controlling microbial infections with reduced side effects becomes indispensable. Hence, since there is a lack of information on the interactions between amoxicillin and tetracycline and the effects of their combination against bacteria of clinical importance, this study was designed to investigate the influence these antibiotics could have on the antibacterial activity of one another.

MATERIALS AND METHODS

Bacterial strain: The bacteria used in this study included Bacillus cereus (ATCC 10702), Staphylococcus aureus (ATCC 6538), Salmonella typhi (ATCC 13311), Acinetobacter calcoaceticus UP, Klebsiella pneumoniae KZN, Proteus vulgaris KZN, Enterococcus faecalis KZN and Staphylococcus aureus OK2c. These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The antibacterial assays were carried out using Mueller Hinton II Agar (Biolab) and broth.

Antibiotics used in this study: Antibiotic powders of Amoxicillin (Duchefa) and Tetracycline hydrochloride (Duchefa) were used. Stock antibiotic solutions were prepared and dilutions made according to the CLSI (Clinical Laboratory Standardization Institute) method or manufacturer’s recommendations (NCCLS, 1997; Richard et al., 2007).

Antibiotic susceptibility testing: Agar diffusion method: Each of the isolates was standardized using colony suspension method. Each strain’s suspension was matched with 0.5 McFarland standards to give a resultant concentration of 1.5x10^9 CFU mL^{-1}. The antibiotic susceptibility testing was determined using the modified Kirby-Bauer diffusion technique (Cheesbrough, 2006) by swabbing the Mueller-Hinton Agar (MHA) (Oxoids UK) plates with the resultant saline suspension of each strain. Wells were then bored into the agar medium with heat
sterilized 6 mm cork borer. The wells were filled with 100 µL of different concentrations (31.25, 62.5 and 125 µg mL⁻¹) of each of the antibiotics taking care not to allow spillage of the solutions onto the surface of the agar. To determine the combinatorial effect of the antibiotics, different solutions containing combined concentrations (31.25, 62.5 and 125 µg mL⁻¹) of tetracycline and amoxicillin were prepared and used. The plates were allowed to stand for at least 30 min before being incubated at 37°C for 24 h (BSAC, 2002). The determinations were done in duplicates. After 24 h of incubation, the plates were examined if there is any zone of incubation (Bauer et al., 1966). The diameter of the zone of inhibition produced by the antibiotic alone and its combination were measured and interpreted using the CLSI zone diameter interpretative standards (CLSI, 2008). Synergism was considered when combinations exhibited inhibition zones increment of 0.5 mm above those produced by the individual antibiotics (Ahmad and Aqil, 2007).

**Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC):** The Minimum Inhibitory Concentrations (MICs) for the two antibiotics under study were determined in duplicate by the macrobroth dilution method in Mueller Hinton broth according to CLSI (Clinical Laboratory Standardization Institute) (Richard et al., 2007). To determine the MICs of each antibiotic, the concentrations used for each of the antibiotics (0.0019-500) µg mL⁻¹ were prepared by serial dilution in Mueller Hinton broth. To determine their combinatorial effects, combinations of different concentrations used in the determination of the MICs of each of the antibiotics were used. The tubes were inoculated with 100 µL of each of the bacterial strains. Blank Mueller Hinton broth was used as negative control. The bacterial containing tubes were incubated aerobically at 37°C for 24 h. Each combination assay was performed two times. The MIC was defined as the lowest dilution that showed no growth in the Mueller Hinton broth. The MBC was defined as the lowest dilution that did not show growth on Mueller Hinton agar after subculturing from the lowest concentrations that showed no bacterial growth (Lorian, 1980).

**Checkerboard assay:** The range of drug concentration used in the checkerboard assay was such that the dilution range encompassed the MIC for each drug used in the analysis. The Fractional Inhibitory Concentration (FIC) was derived from the lowest concentrations of the two antibiotics in combination permitting no visible growth of the test organisms on the Mueller Hinton agar plates after an incubation for 24 h at 37°C (Mandal et al., 2004). FIC indices were calculated using the formula FIC index = (MIC of tetracycline in combination/MIC of tetracycline alone) + (MIC of amoxicillin in combination/MIC of amoxicillin alone). Synergism by the checkerboard method was defined as an FIC index of ≤1, additive effect was defined as an FIC index = 1, Indifference effect was defined as an FIC index of >1 and ≤4 and antagonism effect was defined as an FIC index of > 4 (Kamatou et al., 2006; Petersen et al., 2006). Concentrations within the FIC panel were such that the MIC of each antibiotic was in the middle of the range of concentrations tested but lower than the MICs of the respective antibiotics.

**RESULTS**

In this study, each of the antibiotics as well as their combinations proved inhibitory against all the tested organisms with the exception of *Acinetobacter calcoaceticus* that showed high level of resistance to these antibiotics at the concentrations used singly or in combinations *in vitro* (Table 1). The resistance exhibited by this isolate at the low concentration was corroborated by the result obtained with the minimum inhibitory and bactericidal concentrations (Table 2). A
Table 1: Zones of inhibition resulting from each antibiotic alone and their combinations

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Tetracycline alone (± 1.0 mm)</th>
<th>Amoxicillin alone (± 1.0 mm)</th>
<th>Amx + Tet combined (± 1.0 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125</td>
<td>62.5</td>
<td>31.25</td>
</tr>
<tr>
<td>Bacillus cereus (ATCC 10702)</td>
<td>30</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td>Staphylococcus aureus (ATCC 6538)</td>
<td>23</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Salmonella typhi (ATCC 13311)</td>
<td>32</td>
<td>30</td>
<td>26</td>
</tr>
<tr>
<td>Acinetobacter calcoaceticus UP</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Klebsiella pneumoniae KZN</td>
<td>17</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Proteus vulgaris KZN</td>
<td>17</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Enterococcus faecalis KZN</td>
<td>16</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Staphylococcus aureus OK&lt;sub&gt;28&lt;/sub&gt;</td>
<td>22</td>
<td>19</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 2: Minimum inhibitory and bactericidal concentration values for amoxicillin and tetracycline antibiotics

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Tetracycline (µg mL&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Amoxicillin (µg mL&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Tet/Amx Combined (µg mL&lt;sup&gt;−1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
</tr>
<tr>
<td>Bacillus cereus (ATCC 10702)</td>
<td>0.0305</td>
<td>0.122</td>
<td>7.8125</td>
</tr>
<tr>
<td>Staphylococcus aureus (ATCC 6538)</td>
<td>3.906</td>
<td>7.8125</td>
<td>125</td>
</tr>
<tr>
<td>Salmonella typhi (ATCC 13311)</td>
<td>0.0305</td>
<td>0.061</td>
<td>0.4883</td>
</tr>
<tr>
<td>Acinetobacter calcoaceticus UP</td>
<td>125</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Klebsiella pneumoniae KZN</td>
<td>7.8125</td>
<td>7.8125</td>
<td>31.25</td>
</tr>
<tr>
<td>Proteus vulgaris KZN</td>
<td>7.8125</td>
<td>15.625</td>
<td>0.488</td>
</tr>
<tr>
<td>Enterococcus faecalis KZN</td>
<td>15.625</td>
<td>15.625</td>
<td>0.976</td>
</tr>
<tr>
<td>Staphylococcus aureus OK&lt;sub&gt;28&lt;/sub&gt;</td>
<td>0.9765</td>
<td>1.963</td>
<td>62.5</td>
</tr>
</tbody>
</table>

A comparison of the inhibition zones produced by the combination of tetracycline and amoxicillin with those obtained from each antibiotic showed there is synergistic interaction which is concentration dependent between the two antibiotics. With the exception of Acinetobacter calcoaceticus, inhibitory effects of the combined antibiotics were consistently higher than the inhibitory effects of the individual antibiotic. Staphylococcus aureus (ATCC 6538), Acinetobacter calcoaceticus UP and Staphylococcus aureus OK<sub>28</sub> considered resistant to amoxicillin by having low MIC values were drastically inhibited by the combined antibiotics. While the MIC values for tetracycline ranged between 0.0305 and 125 µg mL<sup>−1</sup>, the MIC values of amoxicillin ranged between 0.061 and 250 µg mL<sup>−1</sup>. Although most of these bacterial isolates had higher MIC values in amoxicillin than in tetracycline, all the bacteria exhibited higher inhibition zones when tested against amoxicillin as compared to tetracycline.

The results of checkerboard assay combinations showed 87.5% synergy and 12.5% antagonism (Table 3). Synergistic interactions between tetracycline and amoxicillin were observed against Bacillus cereus (ATCC 10702), Staphylococcus aureus (ATCC 6538), Salmonella typhi (ATCC 13311), Acinetobacter calcoaceticus UP, Klebsiella pneumoniae KZN, Enterococcus faecalis KZN and Staphylococcus aureus OK<sub>28</sub> (FIC indices of 0.132-0.625) while combinations against Proteus vulgaris KZN exhibited antagonistic interaction (FIC index of 4.25). Although synergy did not depend on the bacterial susceptibilities to each of the antibiotics when used separately but was observed for most of the strains, a high decrease in MIC and a strong bactericidal activity against these strains is of significant importance.
DISCUSSION

Amoxicillin is a broad-spectrum bacteriolytic antibiotic while tetracycline is a broad-spectrum bacteriostatic antibiotic. They are widely used to treat community and healthcare-associated infections. The emergence and dissemination of antimicrobial resistance to these antibiotics represents a significant threat to human health (Woodford and Livermore, 2009). For these antibiotics, numerous resistance mechanisms including expression of drug-destroying enzymes such as β-lactamases (Paterson and Bonomo, 2005), altered drug targets such as conformational changes in penicillin-binding proteins (PBPs), decreased bacterial permeability, increased drug efflux (Wright, 2005; Piddock, 2006; Chen et al., 2011), ribosomal protection and tetracycline modification (Roberts, 2003, 2005) have been described. Discovering a combination therapy capable of preventing or inhibiting the expression of these different mechanisms of resistance will be a giant stride towards effective chemotherapy using these antibacterial agents.

In this study, the impact of each antibiotic on each organism is specific and differs from organism to organism. Amoxicillin and tetracycline showed a powerful bactericidal activity to most of the test organisms and their combinations have apparent synergistic effects. The synergistic effect may be due to the formation of certain complexes which becomes effective in inhibiting the affected microorganisms either by inhibiting the bacterial protein and cell wall synthesis, by causing its lyses or death or by attacking different target sites apart from their known target sites of actions. Bliss (1939) and Greco et al. (1995) indicated that combinations of drugs may inhibit bacterial growth in complex ways, deviating from the neutral situation expected when they do not interact. Hence, the double attack of both agents on different target sites of the bacteria could have theoretically lead to either an additive or a synergistic effect (Esimone et al., 2006) as obtained in this study.

The synergistic interactions resulting in drastic increase in the MICs of the test antibiotics are significant. The ability of these combinations to inhibit Gram-negative and Gram-positive as well as those considered resistant to amoxicillin to a great extent showed that combined therapy of tetracycline and amoxicillin against infectious agents could be more effective than their monotherapy and amoxicillin-clavulanic acid as well as killing pathogens before resistance development. While combined antibiotic therapy has been shown to delay the emergence of bacteria resistance, the synergistic effects are often known to surpass their individual inhibitory activity. In agreement with the report of Sato et al. (2004), the combination of the two agents showed significant synergism because the test organisms had a high MIC to at least one of the agents. The significant increase in the MICs of the antibiotics in the combined state showed that the
combination of the two antibiotics is a veritable source of potential resistance modifying agents (Dickson et al., 2006). Hence, while new therapy can be developed by combining synthetic, semi synthetic and natural drugs in pharmaceutical formulations (Kadam et al., 2010), combining the two antibiotics while reducing their doses and the risk of dose-related toxicity will result in enhanced efficiency (Salam et al., 2009).

Although, there is a dearth of information on the interaction between amoxicillin and tetracycline, interactions between these antibiotics and other chemotherapeutic agents have been reported. Drug-drug interactions between amoxicillin (Wood and Deeble, 1993; Soto et al., 1993; Bandrowsky et al., 1996) as well as amoxicillin-clavulanic acid (Penning-van Beest et al., 2001) with warfarin have been reported in vivo. Most drug interactions of tetracycline are pharmacokinetic and reflect changes in their absorption and elimination or the absorption and elimination of other agents. The plasma concentrations of tetracycline are markedly reduced (30 to 90%) with the concurrent administration of products containing divalent, trivalent and multivalent cations. Tetracycline lowers plasma concentrations of atovaquone and increases that of quinine when concomitantly administered against Plasmodium falciparum (Pai et al., 2006). Vancomycin combined with cephalosporins and penicillins has been shown to synergistically inhibit a number of gram-negative bacilli (Donabedian and Andriole, 1977). Darras-Joly et al. (1996) indicated synergy between β-lactam antibiotics and gentamicin against Streptococcus pneumonia. Olsson et al. (1961) reported antagonistic interactions for penicillin combined with tetracycline against pneumococci, Chan et al. (2007) reported that phenothiazines interact synergistically with amoxicillin against Burkholderia pseudomallei.

In conclusion, these in vitro data still need to be validated by assessing the clinical performances of these combinations. These findings could also prove to be a promising alternative in the treatment of patients for whom existing antimicrobial treatment fails. Despite the lack of knowledge for the underlying mechanism of the synergistic effect of amoxicillin and tetracycline combinations, there is a great potential for use of such combinations against infectious pathogens since it may be able to make some untreatable resistant infections treatable at the recommended dosages that are often marginally effective against resistant strains when used alone. A wider study with increase in the number of drugs and clinical isolates are necessary to establish the mode of action and the mechanism of synergy which is fundamental to the development of a more effective pharmacological agent.

REFERENCES


