Identification and multiple drug resistance of bacteria, isolated from pharmaceutical industrial effluent (Islamabad, Pakistan)

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Abstract

The present study was aimed to determine the Multiple-Drug Resistance among the bacterial strains that were isolated and identified from the effluent of Scottman Pharmaceuticals, Islamabad. Identified strains were Bacillus sp., Klebsiella sp., Coccobacilli, Staphylococcus sp., Enterobacter sp. Unidentified Bacilli, Diplococcus sp., Micrococcus sp., Streptobacillus sp. and Shigella sp. Antibiotic Sensitivity Test was carried out against twenty different antibiotics. Antibiotics used include chloramphenicol (C 10 µg and 30 µg), erythromycin (E 15 µg), ciprofloxacin (CIP 5 µg), cefixime (CFM 5 µg), gentamicin (CN 10 µg), vancomycin (VA 30 µg), streptomycin (S 10 µg), amoxycillin (AMC 30 µg), tetracycline (TE 30 µg), kanamycin (K 30 µg), nitrofurantoin (F 300 µg), ampicillin (AMP 10 µg), rifampicin (RD 5 µg), sulphamethoxazole / trimethoprim (SXT 25 µg), nalidixic acid (NA 30 µg), clindamycin (DA 10 µg), tobramycin (TOB 10 µg), bacitracin (B 10 units), imipenem (IPM 10 µg) and ofloxacin (OFX 5 µg). Results of the study showed that cefixime was the least effective medicine. Maximum resistance was shown by the Staphylococcus sp. (SF21) against ten antibiotics.

Keywords: pharmaceutical effluent, antibiotic sensitivity, Multiple-Drug resistance

Introduction

The pharmaceutical industry embraces of those companies that make drugs and pharmaceuticals that aimed to kill or inhibit disease-causing microorganisms. Pharmaceutical effluents are waste produced by pharmaceutical industry during the process of drugs manufacturing. Effluents released from pharmaceutical industry can be categorized on the basis of type of pharmaceutical compounds such as antibiotics, prescription and non-prescription pharmaceuticals exist in it (Roth et al., 2005). Antibiotics are an important group of pharmaceuticals used in health care for the treatment and prevention of bacterial infections (Kummerer, 2009). Antibiotics can be defined as any substance of natural, semi-synthetic, or synthetic origin that at in vivo concentrations kills or inhibits the growth of microorganisms by interacting with a specific target (Food and Agricultural Organization / Office of International Education / World Health Organization, 2008). The EFSA (European Food Safety Authority) uses the term multiple resistance (MR) or multi-resistance when a bacterial strain is resistant to several different antimicrobials or antimicrobial classes (EFSA, 2008). Antibiotic resistance may spread using various mechanisms, including conjugation, transduction, and transformation (Madigan et al., 2000). The input of resistant bacteria into the environment seems to be an important source of resistance in the environment (Dhundi et al., 2010). The occurrence of
antibiotics can cause selective pressure that favours organisms possessing genes coding for antibiotic resistance. This may pose a serious threat to public health because more and more infections may no longer be curable with known antibiotics (Hirsch et al., 1999). According to CDC (Centres for Disease Control, 1998) antibiotic resistant bacteria cause 2 million deaths each year particularly among individuals with compromised immune systems, including children and the elderly. Antibiotic resistant infections result in longer hospital stay and require the use of more expensive drugs to treat infections (McGowan, 2001). The result of effluent that contains Fluoroquinone antibiotics, when discharged to a river has led to the ability of bacteria to mutate into strains that are resistant to the widely spread antibiotics paving way for infections that cannot be cured (Benotti and Etho, 2005). Gonorrhea was first treated successful with sulfonamides in 1936, but by 1942 most strains were resistant and physicians turned to penicillin. Within 16 years a penicillin-resistant strain had emerged in the Far East (Prescott et al., 2005). There is still lack of fundamental data on the occurrence, fate and effects of antimicrobials in the environment needed for proper risk assessment and risk management both for humans and the environment (Kummerer, 2003).

Materials and Methods

Effluent sample for the research purpose was obtained from the Scottman Pharmaceutical industry Islamabad. The temperature, pH, electrical conductivity and total dissolve solids of pharmaceutical effluent were measured. Bacteria were isolated by serial dilution, and spread plate method. Bacterial colonies were purified with streak plate method. Isolated pure strains were identified on the basis of morphological characteristics and biochemical tests. Colony size, Margins, Forms, Texture, Elevation and Colour was studied. Gram staining and spore staining was also carried out with the help of different dyes. Catalase test, citrate utilization test, gelatin liquefaction test, indole production test, methyl red-Voges Proskur (MR-VP) test, Nitrate Reduction test, starch hydrolysis test, triple sugar iron test and urease test were carried out for the identification. To determine the antibiotic sensitivity of the bacterial isolates, antibiotic-impregnated discs were placed on freshly prepared lawns of each isolate on nutrient agar plates by using Kirby Bauer’s standard antibiotic disk sensitivity testing method (Bauer et al., 1966). The test was performed in triplicates. Discs containing the following antibiotics were tested sulphamethoxazole (25 µg), tetracycline 30 µg, chloramphenicol (10 µg, 30 µg) nitrofurantoin (300 µg), clindamycin (10 µg), erythromycin (15 µg), vancomycin (30 µg), rifampicin (5 µg) and bacitracin (10 units).

Results

Isolated strains from effluent were unidentified bacilli, Diplococcus sp, Streptobacillus sp, Coccobacilli, Micrococcus sp, Gram positive cocci, Gram negative Cocci, Bacillus sp, Staphylococcus sp, Klebsiella sp, Shigella sp. and Enterobacter sp. Most of these species may cause pathogenicity and lead to multiple drug resistance. Results of the study showed that Imipenem and Ciprofloxcin were the most effective medicines and Cefixime was the one that is no more effective now. Maximum resistance was shown by the Staphylococcus sp. (SF21) against ten antibiotics (chloramphenicol (10 µg), erythromycin, cefixime, streptomycin, amoxycillin, nitrofurantoin, ampicillin, sulphamethoxazole, Nalidixic
Table 1. Multidrug resistance of bacterial isolates from effluent collected from pharmaceutical industry

<table>
<thead>
<tr>
<th>Strains</th>
<th>Gram staining</th>
<th>Antibiotics (Resistant/ Sensitive/ Intermediate)</th>
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<tbody>
<tr>
<td></td>
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<td>Amino-glycosides</td>
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<tr>
<td>SF 1</td>
<td>G +ve</td>
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<td>SF 2</td>
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<td>SF 3</td>
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<td>SF 4</td>
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<td>SF 5</td>
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<td>SF 6</td>
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<tr>
<td>SF 7</td>
<td>G -ve</td>
<td>I</td>
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<tr>
<td>SF 8</td>
<td>G +ve</td>
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<tr>
<td>SF 9</td>
<td>G +ve</td>
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<td>SF 10</td>
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<td>SF 11</td>
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<tr>
<td>SF 12</td>
<td>G +ve</td>
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<td>SF 13</td>
<td>G -ve</td>
<td>I</td>
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<tr>
<td>SF 14</td>
<td>G +ve</td>
<td>I</td>
</tr>
<tr>
<td>SF 15</td>
<td>G +ve</td>
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<tr>
<td>SF 16</td>
<td>G -ve</td>
<td>S</td>
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<td>SF 17</td>
<td>G +ve</td>
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<td>SF 18</td>
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<td>SF 19</td>
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<td>SF 20</td>
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<td>SF 21</td>
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<td>SF 22</td>
<td>G +ve</td>
<td>S</td>
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<td>SF 23</td>
<td>G -ve</td>
<td>I</td>
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<tr>
<td>SF 24</td>
<td>G +ve</td>
<td>R</td>
</tr>
</tbody>
</table>

S= Sensitive       I=Intermediate       R= Resistant

* Standards are applicable for only CN, K, S, NA, AMP, AMC, E, V, SXT, CFM, TE, C10, C30, F and B and are characterized as S, I and R. (Source: Bauer et al., 1966).

† Standards for TOB, CIP, IPM, OFX and DA are applicable for only Staphylococcus sp. and Enterobacteriaceae and are characterized as S, I and R. All other species are classified as R and S on the basis of presence and absence of zone of inhibition. (Source: Performance standards for antibiotic disk susceptibility test. Approved standards. 10th edition. Clinical and Laboratory Standards Institute (CLSI), Wayne, P.A.).

RD is classified R and S on the basis of presence and absence of zone of inhibition as its standards were not available.

acid and Bacitracin) and eight classes of antibiotics (Aminoglycosides, Quinolones, β-Lactams, Macrolides, Sulfonamides, Phenicol, Nitrofurans and Polypeptides).

High level of resistance was also shown by Coccid (SF 10), Shigella sp. (SF 6), Diplococcus sp. (SF 9 and SF 24), Streptobacillus sp. (SF 19), Unidentified Bacilli (SF 20) and Bacillus sp. (SF 22) as these strains were resistant against nine, eight and seven antibiotics respectively.

Multiple-Drug Resistance (MDR) was found among SF1, SF 3, SF 4, SF 5, SF 6, SF 7, SF 8, SF 9, SF 10, SF 12, SF 13, SF 15, SF 16 SF 17, SF 18, SF 19, SF 20, SF 21, SF 22, SF 23 and SF 24 bacterial isolates as all these showed resistance against more than two classes of antibiotics.

Discussion

Results from present research showed that bacillus sp, Streptobacillus sp. Diplococcus sp. Cocobacilli, Micrococcus sp., Gram positive Coccid, Staphylococcus sp., Klebsiella sp. Shigella sp. and Enterobacter sp.
surviving in the effluent of the pharmaceutical company. In a microbiological analysis of samples of pharmaceutical facility showed that group of genus, Staphylococci contributes to 38.4% of total positive samples while genus Micrococcus contributes to 22.4% and Gemella morbillorum represents 1.1%. Genus, Bacilli represents 35.0%, Klebsiella pneumonia 1.0% and Stenotrophomonas maltophilia 0.01%. Most microorganisms found among 7 identified genuses were belonging to Staphylococcus, Micrococcus and Bacillus species (Ashour et al., 2011).

The occurrence of bacteria resistant to most of the commonly used antibiotics/drugs is of great medical significance (Khan and Malik, 2001) as of the public health effects. The presence of antimicrobial resistant bacteria has been reported in several studies in polluted and non-polluted environments (Goni-uriza et al., 2000; Mcarthur and Tuckfield, 2000; Hardwood et al., 2000; Costa et al., 2006). The genetic flexibility of bacteria has enabled them to survival in changed environments, because of their capacity to attain and transfer resistance genes. Gene transfer has been observed in aquatic environments (Morinigo, 1990; Angles, 1993). In this study, twenty four bacterial strains were tested for their antibiotic sensitivity against twenty different antibiotics and maximum resistance was shown by Staphylococcus sp. (SF21) against ten antibiotics. Latest reports of Staphylococcus aureus isolates with intermediate or complete resistance to vancomycin threaten a chemotherapeutic period. (Hiramatsu et al., 1997) S. aureus is perhaps the pathogen of greatest concern because of its intrinsic virulence, its ability to cause a diverse array of life-threatening infections, and its capacity to adapt to different environmental conditions (Waldvogel, 2000). Resistance in Staphylococcus aureus mechanisms include enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modification enzymes), modification of the target with reduced affinity for the antibiotic, trapping of the antibiotic (for vancomycin and possibly daptomycin) and efflux pumps (fluoroquinolones and tetracycline). Complex genetic arrays had been developed by S. aureus through horizontal gene transfer, while resistance to other antibiotics, including some of the most recent ones (e.g. fluoroquinolones, linezolid and daptomycin) have developed through spontaneous mutations and positive selection (Pantosti et al., 2007).

The evidence in the another study suggests that antibiotic resistance genes in human bacterial pathogens originate from a multitude of bacterial sources, indicating that the genomes of all bacteria can be considered as a single global gene pool into which most, if not all, bacteria can dip for genes necessary for survival. In terms of antibiotic resistance, plasmids serve a central role, as the vehicles for resistance gene capture and their subsequent dissemination (Bennett, 2008). In study bacteriology of effluents from two pharmaceutical production processes were examined. There bacterial isolates were similar to this research. The bacterial isolates were Staphylococcus aureus, Escherichia coli, Serratia marcescens, Klebsiella sp, Streptococcus pyogenes, Bacillus licheniformis, Yersinia sp., Proteus vulgaris and Bacillus subtilis. Seven patterns of multiple drug resistance ranging from 5 to 11 antibiotics were obtained amongst the isolates (Lateef et al., 2007).

In a study conducted by Lateef et al. (2003) the effluent of a pharmaceutical company was examined microbiologically. The organisms encountered included Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Serratia marcescens and Pseudomonas
The resistances of the 25 bacterial strains isolated from the effluent to the frequently used antibiotics were considered. About 80% of the isolates were resistant to amoxycillin, 76% to nitrofurantoin, 64% to cotrimoxazole and augmentin, 60% were resistant to nalidixic acid, 52% were resistant to tetracycline and ofloxacin, while resistance of 12% was obtained for Gentamicin. Among the eight antibiotics tested, seven patterns of drug resistance were obtained and all of them were multiple-drug resistance. All the strains of *S. aureus* had high MIC values for cloxacillin and amoxycillin. In all, 13 strains of the bacterial isolates had evidence for the production of β-lactamases. Results of this study were in agreement of present study to some extent.

*Shigella* sp. has been acquiring resistance progressively to most commonly used antibiotics as a result of the ability of these organisms to acquire resistance genes located on mobile elements (Sack et al., 1997) and the continuous selective pressure resulting from high levels of antibiotic consumption (Ruiz, 2003). In the present study, *Shigella* sp. has shown resistance against eight antibiotics and intermediate resistance against two antibiotics. In a study Ahmed et al. (2006) reported the genetic characterization of multidrug resistance in *Shigella* sp. Antimicrobial susceptibility tests revealed high levels of resistance, especially to ampicillin, streptomycin, trimethoprim, tetracycline, nalidixic acid and ciprofloxacin. In another study, Ahmed et al. (2006) Pazhani et al. (2008) did molecular characterization of multi drug-resistant *Shigella* sp. in India. *Shigella* species represent one of the growing numbers of antimicrobial-resistant bacteria in developing countries. The majority of the strains harboured genes encoding resistance to ampicillin (97%), tetracycline (95%), streptomycin (95%) and chloramphenicol (94%) (Pazhani et al., 2008).

Some of the strains showed resistance against the aminoglycosides (gentamycin, kanamycin, tobramycin and streptomycin) used in the study. Resistance to aminoglycosides among bacterial isolates has generally been due to acquisition of genes encoding enzymes that modify the structure of specific aminoglycosides (Galimand et al., 2003). High level of resistance (87.5%) of bacterial strains was observed against nalidixic acid i.e 21 strains were resistant for this antibiotic and similar results of high level of resistance to nalidixic acid were reported by Adamu et al. (2009). In the present study four strains showed resistance towards erythromycin. Resistance to erythromycin was first described in 1955 in the United Kingdom (Lowbury et al., 1959). In this study, antibiotics ampicillin, amoxycillin, imipnem and cefixime that include in the β-Lactam class of antibiotics showed 41%, 25%, 0% and 100% of resistance. β-lactam antibiotics remain the most commonly used antibacterial agents, and β-lactamases, the enzymes that hydrolyze β-lactam antibiotics are the major cause of resistance to these compounds (Fisher et al., 2005).

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