ABSTRACT

Introduction

Urinary Tract Infection (UTI) causes inflammation which is a common, painful and sometimes life-threatening condition as well. Despite high prevalence of bacteriuria, the information on biofilm forming bacteria is negligible.

Objectives:

This study aims at understanding the status of the biofilm forming nature of *Klebsiella* spp and *Pseudomonas* spp and their drug resistance property with several class of antibiotics with a prime focus on resistance pattern against few penicillin based drugs to few empirical drugs in today’s me.

Methodology:

Urine samples were analyzed and the isolates were biochemically identified. Then, the isolates were tested for several drugs so as to identify multdrug resistance nature of isolates by Kirby-Bauer Disc method. Biofilm forming nature was examined on Congo Red Agar.

Results

Out of 35 urine samples, 13 isolates (37.1%) were found to be positive with significant bacteriuria. Eight samples (22.8%) showed incidence of *Klebsiella* spp and 5 samples (14.3%) showed *P. aeruginosa*. The prevalence of *Klebsiella* spp. (*Klebsiella pneumoniae* (46.2%) and *K. oxytoca* (15.4%)) and *P. aeruginosa* was found to be 61.4% and 38.6% respectively. 66.6% of *K. pneumoniae* and 50% *K. oxytoca* were biofilm forming pathogen. *K. pneumoniae* and *K. oxytoca* were resistant to amoxycillin, amoxycillin-clavulanate, and cefoxitin; while were sensitive to nitrofurantoin and azithromycin. *P. aeruginosa* were sensitive to azithromycin (100%), but showed 60% resistance to levofloxacin and ofloxacin. Eight (61.5%) isolates were found to be MDR. 100% of *Klebsiella oxytoca* (n=2), 66.7% of *Klebsiella pneumoniae* (n=4), and 40% of *Pseudomonas aeruginosa* (n=2) were multdrug resistant (MDR). Multiple antibiotic resistance (MAR) indices of bacteria revealed that all the 13 isolates were Multi-Antibiotics Resistance strains.

Conclusion

Biofilm forming nature is now much greater in *Klebsiella* spp; while most of the isolates like *Klebsiella* and *Pseudomonas* are multidrug resistant.

KEYWORDS

Antibiotic, biofilm, klebsiella, pseudomonas, susceptibility, uropathogen.
INTRODUCTION

Urinary Tract Infection (UTI) with a pathogen causes inflammation which is a common, distressing and occasionally life-threatening condition. UTI affects people of all ages and both genders. Female are more susceptible to UTIs compared to male. Worldwide about 150 million people are diagnosed with UTIs each year. In sexually active healthy female patients with structurally and functionally normal urinary tracts may have uncomplicated UTIs. Complicated UTIs are associated with co-morbid conditions that prolong the need for treatment like abnormalities of the urinary tract, foreign body presence like indwelling catheter or stone, and infection with MDR pathogens. Signs and symptoms may include fever, chill, dysuria, urinary urgency, frequent and cloudy or malodorous urine.

Infection are almost always ascending in origin and caused by the bacteria inhabiting the distal gastrointestinal tract and colonizing the perineal area. Urinary tract infections are caused by a variety of gram-negative and gram-positive bacteria. The gram-negative bacteria include a large number of aerobic bacilli such as *Escherichia* spp, *Klebsiella* spp, *Pseudomonas* spp, *Enterobacter* spp, *Citrobacter* spp, *Salmonella* spp, *Proteus* spp etc. and the gram-positive bacteria includes *Staphylococcus* spp, *Streptococcus* spp and *Klebsiella* spp. About 80% of acute uncomplicated UTIs are caused by *E. coli*, 10-20% by *Staphylococcus saprophyticus* and 5% or less are caused by other Enterobacteriaceae such as *Klebsiella* spp, *Proteus* spp, or by *Enterococcus* spp. The most common causes of complicated UTIs are *Klebsiella* spp, *Pseudomonas* spp and *Proteus* spp.

Biofilm are complex communities of surface associated cells enclosed in a polymer matrix containing water channels. A biofilm can be composed of a single species or a big conglomerate of microbial species which protects microorganisms from opsonization, antibodies, phagocytosis and removal via the ciliary action of epithelial cell. The emergence of antibiotic resistance in the management of urinary tract infections is a serious public health issue.

In Biratnagar, there are reports of high prevalence of bacteriuria, but information on biofilm forming bacteria is negligible. Based on this dearth, the present study was designed to determine the spectrum of uropathogens in cases of UTI in the Koshi Zonal Hospital (which serves majority of the population of the eastern districts of Nepal) and to determine the antimicrobial susceptibility pattern and biofilm forming nature of the isolates. Our study also aimed at the drug resistance property of pathogens like *Klebsiella* and *Pseudomonas* with a prime focus on resistance against penicillin based drugs to few empirical drugs of today's time.

METHODOLOGY

Study Area and Sample Collection

Clean catch midstream urine samples were collected from both inpatients and outpatients of the Koshi Zonal Hospital, Biratnagar (Nepal). Diversified population (urban and rural) visit the hospital because of low cost treatment by this government hospital. The duration of the study was four months (February-May 2017) and was carried out in the Microbiology Laboratory of the Department of Microbiology at Mahendra Morang Adarsh Multiple Campus (MMAMC), Biratnagar, Nepal. Thirty-five clinical samples of urine were processed during the study. Research and ethical approval were taken from AASRA Research and Education Academy Counsel, Biratnagar. Informed consent was obtained from all participants.

About 15 to 20 ml urine specimen was collected in a 20 ml sterile wide mouthed, screw-capped universal container. The container comprising specimen was appropriately labeled with unique sample number, date, and time of collection. After collection, it was transported to the Microbiology laboratory of MMAMC, Biratnagar for culture, drug susceptibility testing and biofilm formation testing. The specimen was analyzed within two hours of collection.

Sample Culture and Identification of Organisms

Processing of samples were done by a previously described methodology. Patients that presented positive urine culture (≥10<sup>5</sup> CFU/mL) were studied. Significant bacteriuria was defined as colony count ≥10<sup>5</sup> CFU/mL. Each sample was aseptically inoculated (in triplicate) into MacConkey agar plates (Himedia, Mumbai, India) and cetrimide agar plates (Himedia, Mumbai, India). The plates were incubated aerobically at 37°C for 18-24hr. The colonial characteristics of bacterial isolates were observed and sub-cultured.

The resulting isolates were subjected to microscopic examinations like Gram staining, capsule staining and appropriate biochemical tests for proper identification. Gram negative isolates were further identified by different biochemical tests like oxidase, catalase, motility, indole and H<sub>2</sub>S production, MR-VP, citrate utilization, urea hydrolysis, triple sugar iron utilization tests. The identity of bacteria was, thus, established based as described in the book of Cheesbrough.

Biofilm production (Congo Red Agar method)

Congo Red Agar (CRA) method was used for biofilm detection. For this, Congo Red Agar media was prepared with brain heart infusion agar (HiMedia, Mumbai, India) and Congo Red indicator. Congo Red stain was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes separately from other medium constituents. It was, then, added to the autoclaved brain heart infusion agar at 55°C and transferred to the petri-plates. CRA plates were, then, inoculated with fresh isolated pure culture and incubated at 37°C for 24 hours. After incubation, CRA plates were observed whether an organism forms a black colony or not. Well identified *K. pneumoniae* and *Pseudomonas* strains were used as standard. The standard strains were biofilm non-forming and biofilm forming and were used as negative and positive control respectively to compare the result.

Antibiotic Susceptibility Study

Antibiotic susceptibility testing of the bacterial isolates was
achieved by disc diffusion method (Kirby-Bauer method) on Muller-Hinton agar (Himedia, Mumbai, India) and interpreted as per Clinical Laboratory Standard Institute (CLSI) guidelines. A homogenous suspension of 0.5 MacFarland standard of a pure colony was prepared in 5 mL of sterile normal saline (0.85% NaCl). The bacterial suspension was evenly distributed over the entire surface of Mueller-Hinton Agar (MHA) plates using a sterile swab. The antibiotic disc (Himedia, India) containing the following antibiotics was used: Amoxycillin (AMX, 10 µg), Amoxycillin/Clavulanate (AMC) (20/10 µg), Cefotaxime (CTX, 30 µg), Cefoxitin (CFX, 30 µg), Ofloxacin (OF, 5 µg), Levofloxacin (LE, 5 µg), Azithromycin (AZM, 15 µg), and Nitrofurantoin (NIT, 300 µg). Once the discs were applied onto MHA plates, the plates were incubated at 37°C for 24 hr. Zone of inhibition was measured and interpreted using the standard chart and the organisms were reported as susceptible, intermediate, or resistant accordingly. Since Pseudomonas is intrinsically resistant to Amoxycillin, Cefoxitin, and Nitrofurantoin, those antibiotics were not used. Pseudomonas were tested with only five remaining drugs while Klebsiella were tested with eight drugs. Well identified sensive strains of Klebsiella and Pseudomonas were used as control.

The criterion for Multidrug Resistance
All those isolates which demonstrated the resistance to at least one agent in three or more classes of the drug were defined as multidrug resistant (MDR).

Multiple antibiotic resistance (MAR) index
MAR index is a number of antibiotics to which test isolate displayed resistance divided by the total number of antibiotics to which the test organism has been evaluated for sensitivity. So, MAR index for each isolate was calculated as per the recommendation of Krumperman. Data frequencies and cross tabulations were used to summarize descriptive statistics.

RESULT
Isolation and Identification
Out of 35 urine samples, 13 isolates (37.1%) were found to be positive with significant bacteriuria, and no sample showed polymicrobial bacteriuria. Of 35 urine samples, 8 samples (22.8%) showed incidence of Klebsiella spp. and 5 samples (14.3%) showed P. aeruginosa. No growth was seen in rest of the samples. The prevalence of Klebsiella spp. and P. aeruginosa was found to be 61.4% and 38.6% respectively. Eight isolates were from MacConkey Agar plate and 5 were taken from Cetrimide Agar plate. All isolates were identified using cultural, morphological and biochemical characteristics. Upon morphological examination, all the isolates from MacConkey Agar plate when sub-cultured on Nutrient Agar (NA) which upon microscopic examination were found to be capsulated gram-negative bacilli. On the contrary, isolates from Cetrimide Agar plate were non-capsulated gram-negative bacilli. Biochemical test confirmed that 6 isolates \( U_{1}, U_{2}, U_{5}, U_{10}, U_{15}, U_{20} \) of MacConkey plate were Klebsiella pneumoniae, 2 isolates \( U_{15}, U_{30} \) were Klebsiella oxytoca, and 5 isolates \( U_{2}, U_{5}, U_{10}, U_{15}, U_{20} \) were Pseudomonas aeruginosa.

Biofilm formation
Out of 13 isolates, only 5 isolates (4 isolates \( U_{2}, U_{5}, U_{10}, U_{15}, U_{20} \) of K. pneumoniae and 1 isolate \( U_{15} \) of K. oxytoca) formed black colonies on Congo Red Agar plate which showed biofilm forming positive isolates; while all of the Pseudomonas aeruginosa were biofilm non-forming. 66.6% of K. pneumoniae and 50% of K. oxytoca showed biofilm positive.

Figure 1: Biofilm formation by K. pneumoniae

Table 1. Multiple antibiotic resistance (MAR) indices of Klebsiella and Pseudomonas

<table>
<thead>
<tr>
<th>Resistant antibiotics</th>
<th>Number of Drug used</th>
<th>Sample</th>
<th>Strains</th>
<th>MAR</th>
<th>Biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMC + AMC + CFX</td>
<td>8</td>
<td>U2</td>
<td>Klebsiella pneumoniae</td>
<td>0.37</td>
<td>Forming</td>
</tr>
<tr>
<td>AMC + LE + OF</td>
<td>5</td>
<td>U2</td>
<td>Pseudomonas aeruginosa</td>
<td>0.6</td>
<td>Non-forming</td>
</tr>
<tr>
<td>AMC + AMC + CFX + CTX + LE + OF</td>
<td>8 U2</td>
<td>Klebsiella pneumoniae</td>
<td>0.75</td>
<td>Non-forming</td>
<td></td>
</tr>
<tr>
<td>AMC + AMC + CFX + CTX</td>
<td>8</td>
<td>U2</td>
<td>Klebsiella pneumoniae</td>
<td>0.5</td>
<td>Forming</td>
</tr>
<tr>
<td>AMC + AMC + CFX + CTX</td>
<td>8</td>
<td>U15</td>
<td>Klebsiella oxytoca</td>
<td>0.75</td>
<td>Non-forming</td>
</tr>
<tr>
<td>AMC + AMC + CFX + CTX</td>
<td>8</td>
<td>U15</td>
<td>Klebsiella oxytoca</td>
<td>0.75</td>
<td>Non-forming</td>
</tr>
<tr>
<td>AMC + AMC + CFX + CTX</td>
<td>8</td>
<td>U15</td>
<td>Klebsiella oxytoca</td>
<td>0.75</td>
<td>Non-forming</td>
</tr>
<tr>
<td>AMC + CTX</td>
<td>5</td>
<td>U2</td>
<td>Pseudomonas aeruginosa</td>
<td>0.4</td>
<td>Non-forming</td>
</tr>
<tr>
<td>AMC + CTX + LE + OF</td>
<td>5</td>
<td>U2</td>
<td>Pseudomonas aeruginosa</td>
<td>0.8</td>
<td>Non-forming</td>
</tr>
<tr>
<td>AMC + CTX</td>
<td>5</td>
<td>U2</td>
<td>Pseudomonas aeruginosa</td>
<td>0.4</td>
<td>Non-forming</td>
</tr>
<tr>
<td>AMC + AMC + CFX + CTX</td>
<td>8</td>
<td>U2</td>
<td>Klebsiella pneumoniae</td>
<td>0.75</td>
<td>Forming</td>
</tr>
<tr>
<td>AMC + AMC + CFX + CTX</td>
<td>8</td>
<td>U2</td>
<td>Klebsiella pneumoniae</td>
<td>0.75</td>
<td>Forming</td>
</tr>
<tr>
<td>AMC + CTX + LE + OF</td>
<td>5</td>
<td>U2</td>
<td>Pseudomonas aeruginosa</td>
<td>0.8</td>
<td>Non-forming</td>
</tr>
</tbody>
</table>
Out of 13 isolates, 8 (61.5%) were found to be MDR. 100% of Klebsiella oxytoca (n=2) (U1, U2), 66.7% of Klebsiella pneumoniae (n=4) (U3, U4, U5, U6), and 40% of Pseudomonas aeruginosa (n=2) (U7, U8) were multidrug resistant (MDR) as these strains were resistant to at least one antibiotic of three or more classes (Table 1).

Multiple antibiotic resistance (MAR) indices of bacteria revealed that all the 13 isolates had a MARI of greater than 0.2 giving 100% incidence of Multi-Antibiotics Resistance strains. Of all the Klebsiella, 12.5% (n=1) had MARI as 0.37 and 0.5 each, 80% (n=6) had MARI value of 0.56. Of all Pseudomonas, 1(20%) was resistant to 3 drugs (MARI = 0.6) while 2 strains (40%) were resistant to 2 drugs (MARI=0.4) and rest of 40% were resistant to 4 drugs (MARI=0.8) (Table 1).

DISCUSSION
In this study Klebsiella spp. (61.4%) and P. aeruginosa (38.6%) show higher prevalence compared to the work of Fatima et al and Djordjevic et al who reported 24% of Klebsiella and 10.5% of P. aeruginosa respectively. This is probably because Klebsiella forming capsule are able to produce a potent urease which acts on urea to produce ammonia, rendering the urine alkaline. Klebsiella species has a number of virulence factors, including fimbrae, capsule, iron scavenging systems, and urease. Biofilm production and antibiotic resistance is of a great concern in the treatment of disease and infections. Biofilm highly promotes recurrent and persistent infections, which leads to high mortality rates, prolonged treatments and causes high cost in health care services. The result of biofilm production of the uropathogens showed K. pneumoniae has high ability for biofilm production. 66.6% of K. pneumoniae and 50% of K. oxytoca showed biofilm positive. Biofilm producing bacteria showed much greater resistance to antibiotics than their free-living counterparts. This study showed 83.3% of Klebsiella species were biofilm positive while all the isolates of P. aeruginosa were biofilm negative which deviated with the findings of Maharjan et al and Yang who found 20% P. aeruginosa were biofilm positive and 44.7% Klebsiella spp respectively.

This study revealed a higher prevalence rate of resistant to the commonly prescribed antibiotics. Klebsiella were highly resistant to amoxycillin, amoxycillin/clavulanate, cefotaxime and cefoxitin. This could be due to the production of ESBLs capable of conferring bacterial resistant to the penicillin, first, second and third generation cephalosporin such as cefotaxime and cefoxitin. They exhibit resistance to antibiotics by various method like restricted penetration of antibiotics into biofilm, decrease growth rate and expression of resistance genes. The study revealed Pseudomonas aeruginosa were not tested with amoxycillin, cefoxitin, and nitrofurantoin because of their intrinsic resistance towards these drugs. Efflux pumps are common component of multidrug resistant P. aeruginosa and they prevent accumulation of antibiotic within the bacterium, extruding the drugs from the cell before they have the opportunity to achieve an efficient concentration at the site of action. Klebsiella pneumoniae showed 100% resistance to amoxycillin which agreed (100%) with the findings of Beyene and Tsegaye. Resistant to antimicrobial agents include: intrinsic resistance, altered permeability barriers across bacterial outer membranes, preventing uptake of the compounds by inhibiting its corresponding transport carriers, modifying the target’s binding sites so that it no longer recognizes the antibiotics, forming biofilm, mutation and also the ability to enzymatically degrade the antibiotics. Around 61.5% of isolates were found to be MDR. 75% of Klebsiella spp and 40% of Pseudomonas aeruginosawere MDR which was much lower than Singh et al. MARI of all isolates were greater than 0.2 giving 100% incidence of Multi-Antibiotics Resistance strains which was in total agreement with Oli et al.

CONCLUSION
While most of the isolates like Klebsiella and Pseudomonas are multidrug resistant, biofilm forming nature is now much greater in Klebsiella spp.

RECOMMENDATIONS
A regular supervision and broad scale cross sectional study of MDR and biofilm forming have to be done so as to have a clear idea regarding the changes in the nature of bacteria which will guide the medical practitioner about the use of empirical treatment.

LIMITATIONS OF THE STUDY
Due to unavailability of standard strains for quality control, the ATCC standard strains were not used. More sample size might have supported data strongly.

ACKNOWLEDGMENTS
We sincerely appreciate the efforts and inputs of AASRA Research and Education Academy Counsel for making this study possible.

CONFLICT OF INTEREST
The authors declare that they have no competing interests.

FUNDING
No fund/grant was received for the research work.
REFERENCES


