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# Molecular Characterization of Moraxella Catarrhalis and it's Antibiotics Susceptibility Patterns from Different Respiratory Tract Infection Patient's Clinical Samples

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## ABSTRACT

### Introduction

*Moraxella catarrhalis* is a gram negative, oxidase positive, cocci mainly causing upper and lower respiratory tract infections. The RND family efflux pumps lead to multidrug resistance in most of the gram negative bacteria. One of the well- known pumps in *M. catarrhalis* is arcAB and oprM system. The objective of this study was to investigate the antibiotic resistance in *M. catarrhalis* and to determine its resistance dependence on the efflux pump.

### Methods

In this study, 283 different respiratory tract infection samples processed out of that 73 were confirmed by biotyping and molecular characterization as a *M. catarrhalis*. The antibiotic susceptibility tests were performed by disc diffusion method according to CLSI. Molecular characterization for multidrug resistance isolates was also done along with genes responsible for efflux pumps. PCR was done *M. catarrhalis* and acrAB and oprM genes. The factor for association of efflux pump with antibiotic resistance was investigated using phenylalanine argine β-naphthylamide.

### Results

The antibiotics susceptibility result showed 12 out of 73 isolates were MDR, selectively taken for PCR using 16SrRNA specific primer. The MDR isolates were further confirmed by PCR. The amplification results of acra, acrb and oprm genes for the *M. catarrhalis* having multidrug resistance genes by PCR band size products seen on 2% agrose gel. The highest resistance towards the drugs viz penicillin, ampicillin, amoxicillin, amoxiclave, cotrimoxazole, cefazolin, cefuroxime, ceftrixone and cefepime were seen in *M. catarrhalis.*

### Conclusions

The result showed that *Moraxella catarrhalis* is one of the major causes for respiratory tract infection. The drug resistance in this species is increasing day by day. The 90% of isolates have acrAB and oprM genes for efflux pump responsible for multiple drug resistance. The efflux pump inhibitor has important clinical significance for the proper treatment.

**Keywords:** *Moraxella catarrhalis*; AST; PCR; acrAB and oprM; PAbN.

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## INTRODUCTION

*Moraxella catarrhalis* is gram negative diplococci, non-motile and non spore bearing bacteria. Until, 1995 it was considered as a non pathogenic respiratory tract flora. However, this bacteria in a sputum with clinical correlation like increased blood white cell count and C- reactive protein recognized as a human pathogenic agent afterwards.[1] This bacteria is an important pathogen and a common cause of both upper and lower respiratory tract infections, pneumonia, sinusitis and conjunctivitis in infants, children and in elderly patients. In adults, *M. catarrhalis* also causes chronic obstructive pulmonary disease (COPD) and pneumonia.[2] The isolation, identification and antibiotic susceptibility test is not routinely done after taking samples from pharynx, sputum, sinus and also from ear discharge. The treatment of most of the respiratory tract infection is experimental and most of the time it is targeted for three pathogens, namely *H. influenzae*, *M. catarrhalis* and *S. pneumoniae*.[3] Therefore, the response and the resistance patterns against antibiotics has changed dangerously in many treatment centers.

This *M. catarrhalis* has Bro–gene which helps in production of β-lactamases which has moderate effect on antibiotic susceptibility to other β-lactams and cephalosporins.[4] β- lactum drugs are most frequently used drugs in our community which causes drug resistance to β-lactam antibiotics like penicillin. *M. catarrhalis* has another mechanism like exiting efflux pump of group division nodulation resistance (RND) by which it also shows multi drug antimicrobial resistance. This is the mechanism by which bacteria gets protected against different class of antibiotics viz β-lactams, quinolones and aminoglycosides.[5] The genes acrAB and oprM belongs to RND efflux system family in *M. catarrhalis*. The efflux pump has inner membrane pump (acrB), an outer membrane channel (oprM) and a Periplasmic adaptor protein (acrA) which facilitates outer and inner membrane antibiotics transmission in bacteria. There are some groups of drug that elevates the acrAB and oprM genes expression, causes multidrug resistance.[6] Therefore, efflux pump inhibitors has great role for designing novel drugs. Antibiotics resistance in bacteria is a global problem in respiratory tract infections. In Nepal only few research data are available concerning isolation, identification by PCR (efflux pump) and antibiotics susceptibility test of *M. catarrhalis*. Therefore, the present research was aim to molecular identification of efflux pump in *M. catarrhalis* and inhibiting efflux pump by using phenylalanine argine β-naphathalamide (PaβN)[7] responsible for the cause of the disease and their multidrug resistance pattern among the isolates.

## METHODS

In this descriptive cross-sectional research 283 clinical samples, including pharyngeal swabs (n=78), sinus secretions (n=34), sputum (n=171) and nasopharyngeal swab of healthy individuals as control group (n= 100) were also taken from January 2017 to December 2021. The patients were selectively enrolled after taking their consent and following clinical diagnosis by specialist doctor from college of Medical Sciences, Bharatpur, Nepal. The research was approved by institutional ethical review committee. The samples were transferred as soon as possible after collection to the clinical Microbiology department for further analysis. All the samples taken for the research were further processed according to the standard microbiological procedure. The samples were cultivated on Blood Agar and MacConkeys agar and incubated at 37°C for 12-18 hours. After overnight incubation, all the suspected colonies were selectively taken and phenotypically identified using grams stain, catalase test,

oxidase test, nitrate reduction test, DNase test and some sugar fermentation test of glucose, sucrose and lactose. For further confirmation hockey puck test was also applied by taking a wooden stick to push the colonies across the plate.

The antibiotic sensitivity tests of the pathogen isolated from clinical specimen against different antibiotic was done using mueller hinton agar (MHA) (HiMedia) by the standard disk diffusion technique of Kirby-Bauer method.[8] At least two to three well isolates colonies of the same morphotypes were selectively taken from the MHA plate. The base of each colony was touched with an straight wire and the growth were transferred into a tube containing 5ml of nutrient broth for *M. catarrhalis* and then incubated at 37°C (usually 2 to 6 hours) until it achieved turbidity of 0.5 McFarland tube (1.5 x $10^8$ cells $ml^{-1}$). Once turbidity is maintained, 100 ul of bacterial cell suspention was fully streacked on the MHA medium (HiMedia) and antibiotics disc were placed after drying the plate: penicillin (10µl), ampicilllin (10µl), amoxicillin (25µl), cefepime (30µl), ceftriaxone (30µl), ciprofloxacin (5µl), amoxyclave (20µl), gentamycin (10µl), erythromycin (30µl), azithromycin (15µl) and plates were incubated at 37°C for 24 hours. The disc strength and the zone size were interpreted according to clinical laboratory standard institute (CLSI) guideline.[8]

The presence of β-lactamase enzyme and extended spectrum β-lactamases (ESBL) enzyme in antibiotics resistant isolates was determined by the use of nitrosafin and amoxicillin/clavulanic acid discs (HiMedia) respectively.[9] To find out the presence of efflux pump among the *M. catarrhalis*, a compound called as phenylalanine arginine beta naphtylamide (HiMedia) was used as a chemical inhibitor. The culture plates were placed at 37°C for 24 hours. The zone of inhibition on a plate was reported as sensitive, intermediate sensitive and resistant based on CLSI guideline.[8]

### *Morexella catarrhalis* identification by PCR

The multidrug resistance Morexella catarrhalis genomic DNA was confirmed by 16SrRNA sequence as shown in table-1. The DNA was extracted from *Morexella catarrhalis* by using kit (korea). Uniplex PCR was performed by using Morexella catarrahlis primers (16S rRNA) F: (5'-CAG GCC BBB* CAC ATG CAA GTC -3') and R (5'-GGG CGG BBB* GTA CAA GGC-3').[10] The primers and the PCR reagents were purchased from Bangalore Genei Pvt. Ltd., India. The PCR was carried out by using DNA extract assessed at 260 and 280 nm wavelength of the Eppendrof Germany master cycler gradient. The PCR reaction mixture constituted of a final volume 25 µl, which includes 3 µl of deionized distilled water, 1 µl of forward and reverse primer and 2 µl of template DNA. The PCR amplification conditions of an initial denaturation at 95°C for 5 min, followed by 35 cycles including degreasing at 95°C for 20sec, primer binding at 60°C for 30 sec, and a final extension at 72°C for 2 minute.

### Efflux gene (arcA, arcB and oprM) identification from different isolates of multidrug resistant *M. catarrhalis*

Three different specific primers for PCR were used to identify the efflux genes (arcA: F TTG GTT TAG AAG BBB* GTG GC-3, and R: 5-TAG TAT GGT GCA BBB*AGG AG-3, arcB: F 5-ACC ACA BBB* GAG GCA AGT AT-3 and R 5-TGC CGA TGG BBB* TTG TTA AAT-3, oprM: F: 5-CAG GCC TAA BBB* ATG CAA GTC-3 and R: 5-GGG CGG BBB* GTA CAA GGC-3) among different multidrug resistance *M. catarrahlis*. These efflux pump gene primers were designed by using the gene in NCBI and Bioinformatics methods (table-1). The PCR reaction mixture constituted of a final volume 18 µl of Mastermix, which includes 1 µl of forward and reverse

primer, 2 µl of template DNA and 3 µl of deionized distilled water with final volume of 25 µl. The Master mixture was mixed with the efflux pump primers and the tubes containing PCR mixture in a thermocycler (Bio-Rad) with a temperature programme of 15 sec was at first denatured at 95°C and further 35 cycles including degreasing at 94°C for 5 sec, connection at 58°C for 15 sec, and final elongation at 72°C for 2 min were done. The obtained PCR products were then transferred to 2% agrose gel and identified by gel documentation electrophoresis.[11]

*Discloser: *Morexella catarrahlis* primers and Efflux gene (arcA, arcB and oprM) identification primer we don't want to disclose therefore added BBB*.

**Diagnostic sensitivity:** The ten-fold serial dilution was done with the *M. catarrahlis* ATCC 25238, to estimate the diagnostic sensitivity of the PCR assay. Laboratory isolated *E. coli* boiled extract DNA were used to test the specificity of PCR primers as negative control.

**Detection of amplified products:**

**Table 1.** Shows total number of cases from which culture positive *M. catarrhalis* isolated with their percentage values.

| Factors | Number of cases (283) | Culture Positive cases (73) | (Percent) out of 73 cases | p-value |
|---|---|---|---|---|
| **Age distribution** | | | | |
| <20 | 7 | 1 | 1.4 | p <0.2 |
| 21-30 | 22 | 2 | 2.7 | |
| 31-40 | 38 | 6 | 8.2 | |
| 41-50 | 93 | 33 | 45.2 | |
| 51-60 | 76 | 20 | 27.4 | |
| >61 | 47 | 11 | 15.1 | |
| **Sex distribution** | | | | |
| Male | 173 | 45 | 61.6 | p < 0.001 |
| Female | 110 | 28 | 38.4 | |
| **Different samples from which *M. catarrhalis* isolated** | | | | |
| Sputum samples | 109 | 38 | 52.1 | p < 0.001 |
| Pharyngeal swab | 79 | 18 | 24.6 | |
| Sinus secretions | 57 | 11 | 15.1 | |
| Broncho-alveolar levage | 38 | 6 | 8.2 | |
| **Among 73 culture positive *M. catarrhalis* isolated patients had** | | | | |
| Productive cough | 118 | 36 | 49.3 | p < 0.001 |
| Normal chest X-ray | 46 | 5 | 6.8 | |
| Old changes in chest X-ray | 55 | 11 | 15.1 | |
| COPD | | 13 | 17.8 | |
| Interstitial lung disease | 37 | 5 | 6.8 | |
| Lung fibrosis | 27 | 3 | 4.1 | |

The PCR amplified products were elecrophoretically separated in a 2% agarose gel in 1× *Tris* acetate- EDTA buffer and they were visualized by using ethidium bromide, under a UV trans illuminator.[12]

All the data were processed using SPSS version 22. Chi-square test was used to analyze the relationship between the efflux pump and the drug resistance and the independent t-test was used to analyze the relationship between antibiotics and dependent on efflux pump before and after using efflux pump inhibitor. The 95% confidence level was considered for the significance of the tests ($p \leq 0.05$).

## RESULTS

There were 283 respiratory tract infection patient samples were taken for the further isolation of *M. catarrhalis*. Out of them, 73(25.8%) patients had culture and biochemical test positive for *M. catarrhalis* among them, 45(61.6%) were males and 28(38.4%) were females and male female ratio is 1.6:1. The age distribution is listed in table-1 where mean patients age group was 56.4 with a range of 20-80 years. The positive culture isolates were 38(52.1%) sputum samples, 18(24.6%) pharyngeal swab, 11(15.1%) sinus secretions and 6(8.2%) broncho-alveolar levage (BAL).

Among 73 culture positive *M. catarrhalis* isolated patient, 54(73.9%) had productive cough with positive clinical infective marker, 5(6.8%) presented with normal chest X-ray and 11(15.1%) patients had old changes in their chest X-ray. There were 13(17.8%) patients with COPD, 5(6.8%) with interstitial lung disease, 3(4.1%) with lung fibrosis as shown in table-1. Among them 18(24.6%) patient had the history of co-morbid conditions like diabetes, hypertension and ischemic heart disease.

### Antibiotics susceptibility patterns of isolated *M. catarrhalis*

The research result showed out of 73 *M. catarrhalis* 12(16.4%) isolates had highest resistance towards the drugs viz penicillin, ampicillin, amoxicillin, amoxiclave, cotrimoxazole, cefazolin, cefuroxime, ceftrixone and cefepime and least resistace towards the drugs like gentamycin, ciprofloxacin, erythromycin and azithromycin. These 12(16.4%) isolates which showed resistant to two or more classes of drugs were considered as multidrug resistant strain. Among the isolated *M. catarrhalis* all the isolates (100%) were susceptible to gentamycin and ciprofloxacin but 70 (95.9%) were sensitive for azithromycin and 67(91.8%) were sensitive against erythromycin. The current research result also showed that penicillin is the drugs which were (100%) resistance as shown in (Figure 1).

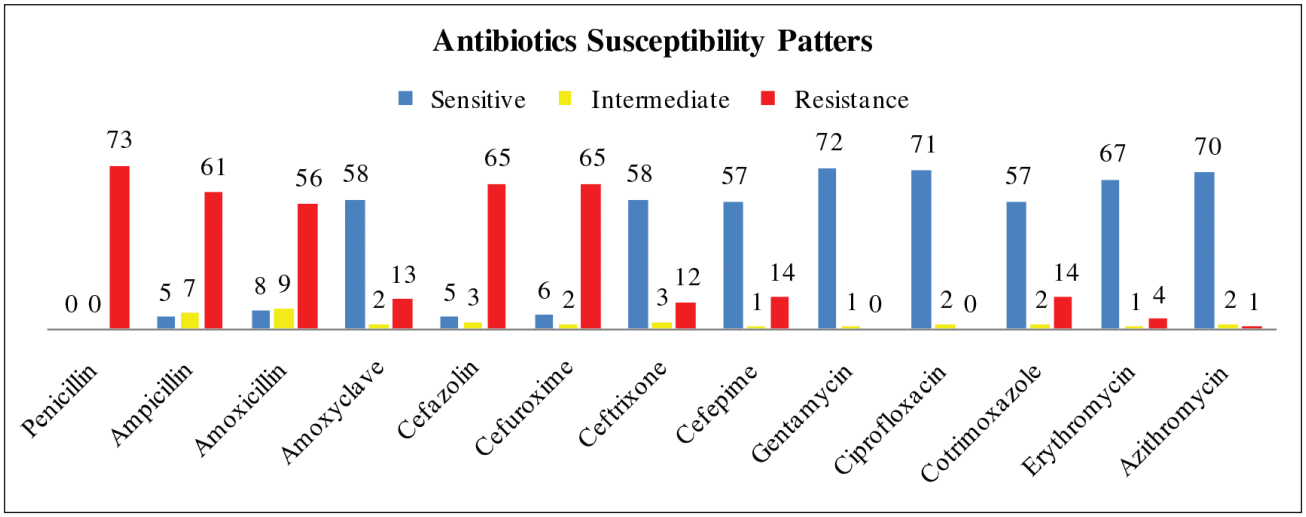


**Figure 1.** Shows the susceptibility patterns of antibiotics against *M. catarrhalis*.

### *Moraxella catarrhalis* isolated by PCR

The antibiotics susceptibility result showed 12 out of 73 isolates were MDR, selectively taken for PCR using 16S rRNA specific primer. The MDR isolates were further confirmed by PCR. The specific band of 1360 base pairs strongly confirmed the presence of *M. catarrhalis* as shown in (Figure 2).

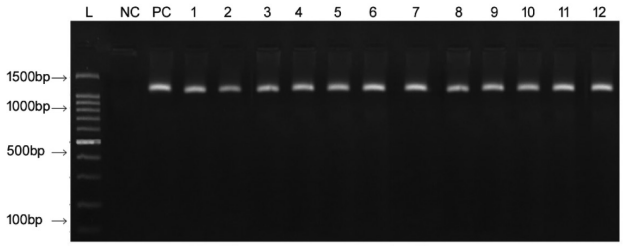


**Figure 2.** Figure-2: 2% Agarose gel electrophoresis showing 16S rRNA of *Morexella catarrhalis* specific PCR on respiratory tract specimens: Lane L: Molecular size marker (100bp), Lane NC: Negative control: *E. coli* (Laboratory isolated) – Negative, Lane PC: Positive control: *Morexella catarrhalis* (ATCC-25238) – Positive, Lane 1-12: Multidrug resistant strain of *M. catarrhalis* of product length (1360bp) – Positive.

### Study of acrA, acrB and oprM genes

The amplification results of acra, acrb and oprm genes for the *M. catarrhalis* having multidrug resistance genes by PCR band size products seen in Figures 3, 4 and 5 on 2% agrose gel. This gold standard test PCR proved the efflux pump genes were present among all the 12 isolates of multidrug resistance *M. catarrhalis*.

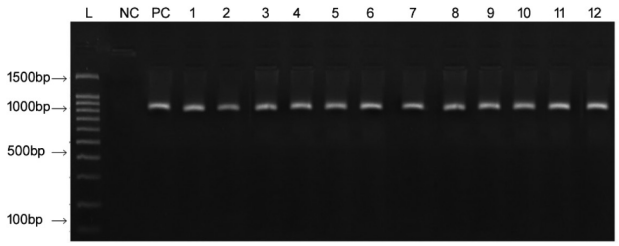


**Figure 3.** Agarose gel 2% electrophoresis showing acra gene of *Morexella catarrhalis* specific PCR on respiratory tract specimens: Lane L: Molecular size marker (100bp), Lane NC: Negative control: *E. coli* (Laboratory isolated) – Negative, Lane PC: Positive control: *M. catarrhalis* (Laboratory isolated) – Positive and Lane 1-12: Multidrug resistant strain of *M. catarrhalis* of product size length (1061bp) – Positive.

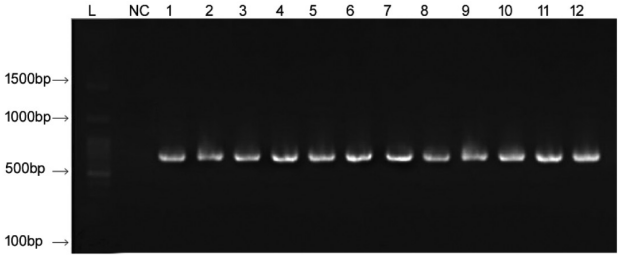


**Figure 4.** 2% agarose gel electrophoresis showing acrb gene of *Morexella catarrhalis* specific PCR done on respiratory tract specimens: Lane L: Molecular size marker (100bp), Lane NC: Negative control: *E. coli* (Laboratory isolated) – Negative and Lane 1-12: Multidrug resistant strain of *M. catarrhalis* of product size length (719bp) – Positive.

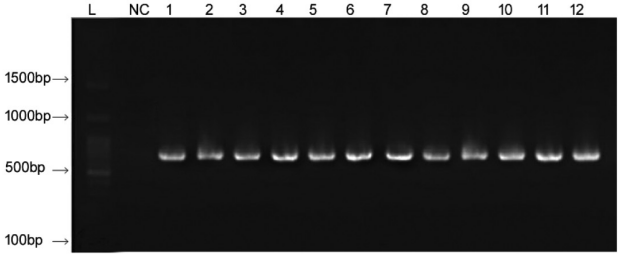


**Figure 5.** 2% agarose gel electrophoresis showing aprm gene of *Morexella catarrhalis* specific PCR done on respiratory tract specimens: Lane L: Molecular size marker (100bp), Lane NC: Negative control: *E. coli* (Laboratory isolated) – Negative and Lane 2-12: Multidrug resistant strain of *M. catarrhalis* of product size length (692bp) – Positive.

The present clinical research was also supported by statistical analysis by using chi-square test, there was clear observed value of P-value<0.05 significance seen between multidrug resistance isolates and the detection of acra, acrb and oprm genes for efflux pump. There were few drugs like amoxiclave, erythromycin, azithomycin and cotrimoxazole did not showed p-value significance.

### Antibiotics susceptibility patterns by using Efflux pump inhibitors

Antibiotics susceptibility patterns showed clear differences, with and without efflux pump inhibitor in the culture medium. The antibiotics like ampicillin, amoxicillin, amoxiclave,



Figure-3:

cefazolin, cefuroxime, ceftrixone and cofepime became more susceptible after addition of efflux pump inhibitor. These isolates with efflux pump inhibitor were clearly showed the zone of inhibition size larger compared to the plates without efflux pump inhibitor. Therefore, the present research shows the exact effect of efflux pump on multidrug resistance against different antibiotics.

genetics and environmental conditions.[14] In the present research, there were 283 respiratory tract infection patient samples were taken for the further isolation of *M. catarrhalis*. Out of them, 73(25.8%) patients had gram reaction, culture and biochemical test positive for *M. catarrhalis*. The positive culture isolates were 38(52.1%) sputum samples, 18(24.6%) pharyngeal swab, 11(15.1%) sinus secretions and 6(8.2%) broncho-

**Table 2.** Antibiotic resistance patterns of M. catarrhalis against different antibiotics based on the CLSI guideline.

| Antibiotics | Without Inhibitors (n/%) | | | With Inhibitors (n/%) | | |
|---|---|---|---|---|---|---|
| | S | I | R | S | I | R |
| Penicillin | 0(0.0%) | 0(0.0%) | 73(100%) | 0(0.0%) | 0(0.0%) | 73(100%) |
| Ampicillin | 5(6.8%) | 7(9.6%) | 61(83.6%) | 25(34.2%) | 11(15.1%) | 37(50.7%) |
| Amoxicillin | 8(10.9%) | 9(12.3%) | 56(76.7%) | 29(39.7%) | 11(15.1%) | 33(54.2%) |
| Amoxyclave | 58(79.5%) | 2(2.7%) | 13(17.8%) | 67(91.8%) | 2(2.7%) | 4(5.5%) |
| Cefazolin | 5(6.8%) | 3(4.1%) | 65(89.0%) | 6(8.2%) | 2(2.7%) | 65(89.0%) |
| Cefuroxime | 6(8.2%) | 2(2.7%) | 65(89.0%) | 54(73.9%) | 5(6.8%) | 14(19.2%) |
| Ceftrixone | 58(79.5%) | 3(4.1%) | 12(16.4%) | 71(79.3%) | 2(2.7%) | 0(0.0%) |
| Cefepime | 57(78.1%) | 1(1.4%) | 14(19.2%) | 72(98.6%) | 1(1.4%) | 0(0.0%) |
| Gentamycin | 72(98.6%) | 1(1.4%) | 0(0.0%) | 73(100%) | 0(0.0%) | 0(0.0%) |
| Ciprofloxacin | 71(79.3%) | 2(2.7%) | 0(0.0%) | 73(100%) | 0(0.0%) | 0(0.0%) |
| Cotrimoxazole | 57(58.1%) | 2(2.7%) | 14(19.2%) | 57(58.1%) | 2(2.7%) | 14(19.2%) |
| Erythromycin | 67(91.8%) | 1(1.4%) | 4(5.5%) | 69(94.5%) | 1(1.4%) | 3(4.1%) |
| Azithromycin | 70(95.9%) | 2(2.7%) | 1(1.4%) | 71(79.3%) | 1(1.4%) | 1(1.4%) |

## DISCUSSION

Respiratory tract infection is mostly caused by *Hemophilus influenza, Streptococcus pneumonia, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Morexella catarrhalis* species. Among them *M. catarrhalis* is also responsible for most of the respiratory tract infection in healthy and immunocompromised patients because it has important virulence factors like receptors sites for attachment on cellular component and mucous, production of enzymes and capability to form biofilm.[13] There are many factors and co-factors which increases the chances of *M. catarrhalis* infectivity like age, sex, nutritional status, hygiene condition, immune status, alveolar levage (BAL). In a year 2016 study done by Sillanapass *et al.* found out of 222 respiratory samples only 22 *M. catarrhlis* was isolated, where as other researcher found that out of 48 chronic obstructive pulmonary disease only eight i.e (9.0%) were *M. catarrhalis* as a main cause of the disease.[15,16] The given values are not consistent with our result, our result showed higher value (25.8%). However, the marked increased value differences were seen because of the prevalence of bacteria and unhygienic condition of the patients in this geographical area.

In the present study, *M. catarrhalis* was identified by its ability to grow in nutrient agar at 35°C, hydrolysis of tributyrin and DNase tests. These

results were similar to that of Verduin *et al.*[17] result who also found phenotypic identification is useful for species identification but further confirmation requires molecular technique. According to the age distribution we found mean patients age group was 56.4, which ranges from 20-80 years. The highest number 33(45.2%) of *M. catarrhalis* was isolated from 41-50 age group of patents. Among them, 45(61.6%) were males and 28(38.4%) were females and male female ratio is 1.6:1. Our result was in alignment with the previous report, which also supported that male with 41-50 years of age are more liable to get infection by *M. catarrhlis*.[18] This situation occurs due to male works outside the house for many other purposes compared to female patients.The result of Kirby-Bauer disc diffusion for antibiotics susceptibility test pattern of *M. catarrhalis* isolates against different antibiotics with high resistance to β- lactam antibiotics. The research result showed out of 73 *M. catarrhalis* 12(16.4%) isolates had highest resistance towards the drugs *viz.* penicillin, ampicillin, amoxicillin, amoxiclave, cotrimoxazole, cefazolin, cefuroxime, ceftrixone and cefepime and least resistance towards the drugs like gentamycin, ciprofloxacin, erythromycin and azithromycin as shown in figure-1. This type of susceptibility patterns shown by isolates is due to its ability to produce β-lactamases enzymes. Shi *et al.* also isolated bacteria producing b-lactamases showing resistant to penicillin, and susceptible to antibiotics viz erythromycin, tetracycline, cotrimoxazole and amoxiclave.[19] In the present research we found only 12(16.4%) isolates which showed resistant to two or more classes of drugs were considered as multidrug resistant strain. Among the isolated *M. catarrhalis*, they were (100%) susceptible to gentamycin and ciprofloxacin antibiotics. Therefore, these antibiotics are the best for the proper treatment of infection caused by M. catarrhlis. Similar pattern of susceptibility was also delineated by Krishna et al. and Gergova *et al*.[20,21] But we have also found 70 (95.9%) isolates were sensitive for azithromycin and 67(91.8%) were sensitive against erythromycin. Alternatively, azithomycin and erythromycin are the drugs which also can be used in the treatment of respiratory tract infections in developing countries like Nepal. The current research result also showed that penicillin is the drugs which were (100%) resistance. Our research report also corroborates with the result of Shaikh *et al.* who found *M. catarrhalis* was highest resistance to penicillin (100%).[22] Mohammad *et al.* examined 200 patients, of whome 91.3% were resistance to penicillin and 100% susceptible to amoxiclave and 97.7% to tetracycline.[23]As we have seen there are many drugs which are getting resistance among the *Morexella* species are big burden for treatment in community as well as in hospital settings. One of the main causes for the increase in resistance in these days is due to arbitrary use, over the counter medication and partial treatment for shorter period of time. The antibiotics susceptibility result showed 12 out of 73 isolates were multidrug drug resistance, selectively taken for PCR using 16SrRNA specific primer. The MDR isolates were further confirmed by PCR as shown in figure-2. The specific band of 1360 base pairs strongly confirmed the presence of *M. catarrhalis*. PCR is the scale which also judges the result of phenotypic characterization of *M. catarrhalis*. That is why; it is also known as a gold standard technique for the microbial species identification. The efflux pump in *E. coli* is similar as that of efflux pump in *M. catarrhalis* having acr-ab and opr-m genes for multidrug leakage system.[24] This experiment shows the amplification results of acra, acrb and oprm genes for the *M. catarrhalis* having multidrug resistance genes by PCR band size products seen in figures - 3, 4 and 5 on 2% agrose gel. This gold standard test PCR proved the efflux pump genes were present among all the 12 isolates of multidrug resistance *M. catarrhalis*. The present clinical research was also supported by statistical analysis by using chi-square test, there was clear observed value of P-value<0.05 significance seen between multidrug resistance isolates and the detection of acra, acrb and oprm genes for efflux pump. There were few drugs like amoxiclave, erythromycin, azithomycin and cotrimoxazole did not showed p-value significance.

According to the result of genotypic characterization of efflux pump and its

involvement in the antibiotics resistance were clearly confirmed in present clinical investigations. Research also shows that there are many number of efflux pump in *M. catarrhalis* due to the increased resistance in a *M. catarrhalis* increases the respiratory tract infections.[24] In another experiment, it has also shown that after treatment with amoxicillin, purine M35 is negatively regulates the efflux pump causing increased drug resistance in isolates.[24] This type of mechanism shows the resistance to aninopenicillins in *M. catarrhalis*. Gram negative bacteria has phenyalalanine argine β-nephthylamide dihydrochloride (PAbN) inhibitor as a first inhibitor of RND pumps.[5] use of this efflux inhibitor in bacteria increases the bacterial susceptibility and also bactericidal activity.[5] Antibiotics susceptibility patterns showed clear differences, with and without efflux pump inhibitor in the culture medium. The antibiotics like ampicillin, amoxicillin, amoxiclave, cefazolin, cefuroxime. ceftrixone, cofepime became more susceptible after addition of efflux pump inhibitor. These isolates with efflux pump inhibitor were clearly showed the zone of inhibition size larger compared to the plates without efflux pump inhibitor. Similar type of results were also observed in *A. baumannii* Owlia *et al.* showed susceptibility to imipenem was increased in the presence of PAbN compound. In another study Rafiei *et al.* showed the combined effect of pump inhibitor in *A. baumannii* by using PAbN and NMP. As seen in present research the antimicrobial properties of some antibiotics with efflux pump inhibitor increases but no effect was observed in aminoglycosides. The present research shows the exact effect of efflux pump on multidrug resistance against different antibiotics which are also similar with above researcher reports. Therefore, bacterial drug resistance and role of efflux pump requires continuous monitoring this will help in the discovery of new drugs for the future use against these different bacterial isolates.

## CONCLUSIONS

There was significant number of *M. catarrhalis* phenotypically isolated and further genotypically confirmed from the patients with respiratory tract infections. The significant high resistance number of isolate was found against β-lactum antibiotics. It is primary alarming sign for respiratory tract infected patients. Hence, continuous research, proper full coarse treatment and reducing over use of antibiotics required to reduce multidrug resistance among pathogenic gram negative bacteria.

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**Conflict of interest:** None.

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