Ventilator Associated Pneumonia in Tertiary Care Hospital, Maharajgunj, Kathmandu, Nepal


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Abstract

Introduction: Ventilator Associated Pneumonia (VAP) is the most common nosocomial infection among intensive care unit (ICU) patients and lack of much information in Nepal. So, the aim of this study was to determine prevalence and bacteriological profile of VAP with special reference to multi-drug resistant (MDR), Methicillin-resistant Staphylococcus aureus (MRSA), Metallo-β-Lactamase (MBL), Extended-Spectrum β-Lactamase (ESBL)-producing bacterial strains.

Methods: A total 150 tracheal specimens were studied during June 2011 to May 2012 at Department of Microbiology, TUTH as described by American Society for Microbiology (ASM). Combination disk method was done for the detection of ESBL and MBL producing isolates.

Results: Prevalence of VAP was found to be 34%. Acinetobacter calcoaceticus baumanniicomplex (44%) was the commonest isolate, followed by Klebsiella pneumoniae (22%), Pseudomonas aeruginosa (16%) and Staphylococcus aureus (12%). Among MDR Gram negative bacteria (GNB), 39% were MBL and 33% were ESBL-producers. All GNB (61) were sensitive to Polymyxin B and Colistinsulphate, whereas, 48% were found resistant to Carbapenems. Prevalence of MRSA was 75%, which were all sensitive to Vancomycin.

Conclusion: High prevalence of VAP, MDR along with MRSA or ESBL or MBL producing strains was found in the study. Thus, suitable control measures must be adopted to cope up this alarming situation with genetic characterization.

Key words: VAP, ICU, MDR, MRSA, ESBL, MBL.

Introduction

People with life-threatening injuries and illnesses need critical care and mechanical ventilation is must. It is often a life-saving intervention, but carries many potential complications, including pneumothorax, airway injury, alveolar damage, collapsed lung and ventilator-associated pneumonia. Ventilator-associated pneumonia (VAP) is defined as an episode of pneumonia in a patient who requires a device to assist or control respiration through a tracheostomy or endotracheal tube at the time of or within 48 hours before the onset of the infection. Eighty-six percent of nosocomial pneumoni as are associated with mechanical ventilation. This is associated with increases in morbidity and mortality, hospital length of stay, and costs.

In modern medical practice, extensive use of antibiotics have resulted in emergence and rapid dissemination of Multi drug resistant (MDR), Methicillin Resistant Staphylococcus aureus (MRSA), Extended-Spectrum β-Lactamase (ESBL) and Metallo-β-Lactamase (MBL) producing bacteria. Thus, their detection is crucial for the optimal treatment of patients and to control the spread of resistance. So this study is intended to address the issues regarding the prevalence of VAP, MDR, ESBL-, MBL-producing bacterial isolates, and MRSA.
Methods

A Laboratory based Prospective study was conducted in Department of Microbiology, Tribhuvan University Teaching Hospital (TUTH), Kathmandu from June 2011 to May 2012. This study was approved by Institutional Review Board of Institute of Medicine. Data were analyzed using SPSS, version 17.0. A total of 150 tracheal secretions received for culture and sensitivity in the laboratory were included in the study. The specimens were cultured on Chocolate agar (CHA), 5% Sheep Blood agar (BA) and MacConkey agar (MA) (Oxoid, UK) plates. On the CHA, bacitracin disk (10 Unit) and optochin disk (5 μg) (Oxoid, UK) were placed at primary and secondary inoculation to screen H. influenzae and S. pneumoniae respectively.

The CHA plates were incubated in CO2 incubator (10% CO2) at 37°C for 24 hours while BA and MA plates were incubated at 37°C for 24 hours in aerobic atmosphere.

Determination of Bacterial Etiology of VAP:

The etiology of VAP was determined as growth of ≥ 106 cfu/ml in endotracheal aspirate and compatible with Gram’s stain result of the specimen.

Identification of isolated organisms:

Firstly, pure form of the culture was obtained from the primary culture by using purity plate and then it was processed for different biochemical tests following standard microbiological procedures.

Antibiotic susceptibility testing:

The susceptibility test of the pathogens isolated from the clinical specimens against different antibiotics was done by the standard disk diffusion technique of Kirby-Bauer method as recommended by Clinical and Laboratory Standards Institute (CLSI). 4 CHA and BA were used for H. influenzae and S. pneumoniae respectively to perform sensitivity test. S. aureus ATCC 25923, E. coli ATCC 25922 and P. aeruginosa ATCC 27853 were also tested in every set of experiment, in parallel, as a part of quality control. In this study if the isolates were resistant to at least three classes of first-line antimicrobial agents, they were regarded as MDR. 5

Tests for ESBL- production in Gram negative isolates

The initial screening test for the production of ESBL was performed by using Ceftazidime (CAZ) (30 mg) and Cefotaxime (CTX) (30 mg) disks (Mast U.K.). If the zone of inhibition was between ≤ 22 mm for Ceftazidime and between ≤ 27 mm for Cefotaxime, the isolate was considered as a potential ESBL producer as recommended by CLSI. Confirmations of ESBL producing strains were done by Combination Disc (CD) method in which CAZ and CTX alone and in combination with clavulanic acid (CA) (10 μg) were used. An increase ZOI of ≥ 5 mm for either antimicrobial agent in combination with CA versus its zone when tested alone confirmed ESBL.

Tests for MBL-production in Gram-negative isolates

Screening test:

The isolates were subjected for MBL detection when the zone of inhibition (ZOI) was < 18 mm to imipenem (IPM) (10 mg) and/or meropenem (MEM) (10 mg). A suspension of bacteria equivalent 0.5 McFarland standard was prepared and was swabbed on to MHA plate.

Combination disk (CD) method:

Two IPM disks (10 μg), one containing 10 μl of 0.1 M (292 μg) anhydrous Ethylenediamine-tetraacetic acid (EDTA) (Sigma Chemicals, St. Louis, MO), were placed 25 mm apart (center to center). An increase in zone diameter of > 4 mm around the IPM-EDTA disk compared to that of the IPM disk alone was considered positive for an MBL.

Tests for MRSA

Thirty microgram cefoxitin disk method as recommended by CLSI was put up and agar plates were incubated at 35°C. The diameter of the zone of inhibition of growth were recorded and interpreted as susceptible or resistant by the criteria of CLSI. S. aureus strains ATCC 25923 were used as negative and positive controls respectively. Organisms were deemed methicillin resistant when the zone of inhibition ≤ 21 mm for S. aureus with cefoxitin disk method.

Results

Number of specimens and result pattern: A total of 150 tracheal secretions were received in the bacteriology laboratory for culture and sensitivity from June 2011 to May 2012. Among the total processed specimens (n=150), significant bacterial growth was found in 64 (42.66%) aspirates, out of which 51 (34%) aspirates were associated with VAP as shown in Table 1.
Ventilator Associated Pneumonia

Table 1: Pattern of Tracheal Aspirates Culture result

<table>
<thead>
<tr>
<th>Growth Pattern</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>No growth</td>
<td>86</td>
<td>57.33%</td>
</tr>
<tr>
<td>Significant growth</td>
<td>64</td>
<td>42.66%</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>100%</td>
</tr>
<tr>
<td>VAP Growth</td>
<td>51</td>
<td>34%</td>
</tr>
<tr>
<td>Non-VAP Growth</td>
<td>13</td>
<td>9%</td>
</tr>
<tr>
<td>Total</td>
<td>64</td>
<td>43%</td>
</tr>
</tbody>
</table>

Among 51 VAP cases, 37 were of Late Onset type.

Pattern of Bacterial isolates

A total of 69 bacterial isolates were isolated from 51 different tracheal aspirate samples. Out of 69, 61 (88.4%) isolates were Gram negative (P<0.01) and remaining (11.59%) were found Gram positive isolates. The commonest isolate causing VAP was Acinetobacter calcoaceticusbaumanni complex (n=30, 43.47%) (P<0.01), followed by Klebsiella pneumoniae (n=15, 21.73%), Pseudomonas aeruginosa (n=11, 15.94%) and Staphylococcus aureus (n=8, 11.59%) (Table 2)

Table 2: Pattern of Bacterial isolates from VAP cases (n=51)

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram negative bacteria</td>
<td>(n=61)</td>
<td></td>
</tr>
<tr>
<td>Acinetobacter calcoaceticusbaumanni complex</td>
<td>30</td>
<td>43.47%</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>15</td>
<td>21.73%</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>11</td>
<td>15.94%</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>3</td>
<td>4.34%</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>2</td>
<td>2.89%</td>
</tr>
<tr>
<td>Gram positive bacteria</td>
<td>(n=8)</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>8</td>
<td>11.59%</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>100%</td>
</tr>
</tbody>
</table>

Antibiogram of Acinetobacter calcoaceticusbaumanni complex

Major VAP isolate i.e., Acinetobacter spp. were found resistant to wide range of antibiotics. Ninety seven percent (n=29) isolates were found resistant to Amikacin and Cefipime, while 83.33 (n=25) isolates were found resistant to Imipenem and Meropenem. However, none of the isolates were found resistant to Polymyxin B and Colistin sulphate.

Antibiogram of Klebsiella pneumoniae

Klebsiella pneumoniae, the second commonest isolates were also found resistant to number of antibiotics. Seventy-three percent (n=11) isolates were found resistant to Ciprofloxacin, Ofloxacin, Ceftriaxone and Cefotaxime, and 67% isolates were found resistant to Amikacin and Cefipime. However, majority of Klebsiella pneumoniae isolates (n=12, 80%) were found sensitive to Meropenem and Imipenem and all the isolates (n=15, 100%) were found sensitive to Polymyxin B and Colistin sulphate. (Figure 3)

4.19: Antibiogram of Pseudomonas aeruginosa

Antibiotic resistance was also found common in Pseudomonas aeruginosa isolates. Predominant isolates were found resistant to Ciprofloxacin, Amikacin, Cefipime and Piperacillin-tazobactam. However, isolates sensitive to Cefoparazon-sulbactam and Carbapenem were found to be 82% and 91% respectively. Likewise, all the isolates were found sensitive to Polymyxin B and Colistin sulphate. (Figure 4)

Antibiogram of Staphylococcus aureus (n=8)

Majority of Staphylococcus aureus (75%) isolates were found resistant to many antibiotics. However, none of the isolates were found resistant to Vancomycin.

MDR, MBL and ESBL producing Gram negative isolates

Many of the Gram negative isolates were found resistant to number of antibiotics. Eighty percent Gram negative isolates were found to be MDR, which was more common among Acinetobacter, Escherichia and Klebsiella isolates.

Among the MDR Gram negative isolates, 39% were MBL producer and 33% were ESBL producer. MBL producing isolates were the most common among Acinetobacter spp. (67%).

Enterobacteriaceae isolates were found to be common ESBL producer. (Figure 1,2)

Figure 1: Pattern of MDR, MBL and ESBL producing Gram negative isolates
Among 150 total tracheal aspirates, 64 samples showed significant bacterial growth among which, 51 samples were associated with VAP. In this study, incidence of VAP was found 34% which was similar to findings of Ranjit S et al (31.88%), Dhulikhel hospital, Nepal, 2011. Lower incidence of VAP was found in Saifdar N et al., (22.8%), 2005, study. Higher incidence of VAP was found among the mechanically ventilated patients in following authors studies; Gadani et al (37%, Gujarat, India 2010), Dey et al (45.45%), Petdachai W et al (49.4%, Thailand, 2004), Kanafani ZA et al (47%, Beirut, Lebanon, 2003), Jakribettu RKP et al (44.2%).

The observation that more than one causative pathogen associated with VAP has been demonstrated in this study. Out of 51 VAP episodes, 35 (68.63%) episodes were monobacterial and 16 (31.37%) were mixed bacterial. In study Visscher et al study, out of 153 VAP episodes 107 (69.9%) episodes were monobacterial and 46 (30%) were caused by two pathogens.

Significant number of isolates were found Gram negative bacilli (GNB) (n=61, 88.4%) and remaining isolates were Gram positive cocci (GPC) (n=8, 11.59%), (P<0.01).

Similar type of findings were found in Koirala P et al (GNB= 89.6%, GPC=10.4%) study from Neuro Hospital, Nepal. In Visscher et al study, 72.37% isolates were GNB and 27.63% isolates were GPC, and 83% GNB was found in Kanafani ZA et al study.

The aetiological agents associated with VAP in this study were Acinetobactercalcoaceticusbaumannamic complex, Klebsiellapneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli and Citrobacterfreundii. These isolates were also common in George P et al Manglore, India study. However, more common pathogens were associated with VAP in Visscher S et al study in which Haemophilusinfluenzae, Streptococcus pneumoniae, Proteus spp., Enterobacteriaceae, Serratia p., Morganellamorgani, Stenotrophomonasmaltophilia were additional isolates. Burkholderiacepacia complex was also found common in European and American VAP pathogens. The aetiological agents of VAP vary with different patient populations and types of ICUs. The causative organisms vary with the patients' demographics in the ICU, the method of diagnosis, the duration of hospital stay, and the institutional antimicrobial policies. VAP may be caused by a wide spectrum of bacterial pathogens. Therefore, the local microbial flora causing VAP needs to be studied in each setting to guide more effective and rational utilization of antimicrobial agents.

In this study, non-fermenters (59.1%) were the major pathogens associated with VAP and remaining isolates were enteric gram-negative bacilli (28.96%) and Staphylococcus aureus (11.59%). Number of studies show that non-fermenters are major VAP pathogens but their incidence rate varies in different setting and geography. In Trouillet JL et al (France, 1998) study, frequency of non-fermenters and Enterobacteriaceae were 33.9% and 17.9% and in Esperati M et al (Spain, 2010) studies, 28% were Non-fermenters and 26% were enteric Gram-negative bacilli.

Acinetobactercalcoaceticusbaumannamic complex was the major pathogen responsible for Early Onset as well as Late Onset Type of VAP. (P<0.01). However, in most of the studies, high rates of H.influenzae, S. pneumoniae, MSSA, or susceptible Enterobacteriaceae were constantly found in Early Onset VAP, whereas P. aeruginosa, Acinetobacterspp.
MRSA, and multiresistant GNB were significantly more frequent in Late-Onset VAP e.g Joseph et al study. Non-fermenters colonization during intra-ward admission period before shifting to ICU-MV may be the reason behind Acinetobactersppas common Early Onset VAP pathogens in this study.

*Acinetobacter baumannii* complex (43.47%) was found to be the most common VAP isolate. However, in earlier studies, *Pseudomonas* spp. used to be the most common ICU pathogens. In the global aspect, there has been increasing concern regarding the rise of Acinetobacter infection, ranging from 4-44% in Asian hospitals and 0-35% in European hospitals. These increasing patterns of Acinetobacter infection which usually have high mortality rate, has alarmed us that there is further need of extensive study and apply preventive measures to reduce such fearful threat from *Acinetobacter* infection in ICU patients.

In this study, there was high prevalence of MDR Gram negative isolates (80.33%) and Gram positive isolates (75%). Frequency of MDR in Joseph NM et al study was 78.7% (Pondicherry, India, 2010), which is similar to this findings. MDR was found prevalent in all types of bacterial isolates. Out of 30, 29 (96.66%) isolates of *Acinetobacter baumannii* complex and 10 isolates out of 15 Klebsiella pneumoniae were found MDR. Among 8 *Staphylococcus aureus*, 6 (75%) isolates were MDR. All *Escherichia coli* (n=3/100%) isolates were found MDR. As there was relatively less number of *E. coli* isolate as compared with other common Gram negative isolates this figure may not reflect the true scenario. Of the 250 isolates of *Acinetobacter spp.*, 88.4% were MDR in Sweih NA et al study. This study clearly explores that the MDR is common in almost all type of Gram negative as well as Gram positive VAP bacteria complicating the treatment of patients. The emergence of MDR pathogens can be prevented by adopting an antibiotic institutional policy and dose de-escalation regimens.

Drug resistance was found common in the all groups of antibiotics commonly being used. Ninety seven percent (n=29) isolates of Acinetobacter spp. were found resistant to Amikacin and Cefipime, and 83.33% isolates (n=25) were found resistant to Cefoparazone–sulbactam and Carbapenem. In the study conducted by Xie DS et al, China, the frequencies of Imipenem-resistant *A. baumannii* was 80.3% which was similar to this study. Following resistance frequencies were found in George et al, Manglore, India, study [Amikacin (55.34%), Imipenem (66.67%), Meropenem (75%) and Cefaparazone (75%)]. This shows that major VAP isolate resistant to most potent and major reserved drugs.

In this study multi-drug resistance was also found common in *K. pneumoniae*, the second most common VAP isolate. Isolates resistant to fluoroquinolones and third generation cephalosporins were 73.33%, to aminoglycosides were 67% and to Cefoperazone-sulbactam were 53.33%. Highly resistant Cephalosporin (Cephalaxin 75%, Ceftriaxone 85%, Cefotaxime 82.5%) in Amin et al study was similar to my study. However, in this study 20% isolates were resistant to carbapenems. This was similar to George et al study, where Klebsiella was found resistant to Meropenem (20%) but higher than Amin et al study, where carbapenems (Imipenem, Meropenem) with the least resistance at 7.5%.

Sixty four *Pseudomonas* isolates were found resistant to fluoroquinolones, 54.54% isolates resistant to Amikacin, 45.45% isolates to Cefipime, 36.36% isolates were found resistant to Piperacillin-tazobactam. However, there was a low resistant frequency (9.1%) to Imipenem/Meropenem/Cefoparazone-sulbactam. In George P et al study, frequencies of Pseudomonas isolates resistance to Amikacin/Piperacillin/Cefoparazone were 14.29%, to Ceftriaxone were 28.58%, and to Imipenem/Meropenem were 42.86%. In a continuous, prospective, multicentre cohortstudy in Hubei Province, China, by Xie DS et al from January 2007 to June 2009, the frequencies of Imipenem-resistant and Ciprofloxacin-resistant *P. aeruginosa* were 42.0% and 58.6% respectively.

In this study, Polymyxin B and Colistinsulphate showed excellent effect against all MDR Gram-negative isolates. Except for *Acinetobacter baumannii* complex even Imipenem and Meropenem were found to be effective against Gram-negative VAP isolates. Other antibiotics found to be effective were Cefoparazone-sulbactam and Piperacillin-tazobactam in decreasing order for other than *Acinetobacter baumannii* complex. Other antimicrobials like Amikacin, Cefipime, Ciprofloxacin, Ofloxacin, and Ceftriaxone showed poor effect among the Gram-negative isolates. High antibiotic resistance rate against commonly used antibiotics is a disadvantage for health care system in countries like Nepal as it can greatly effect patient management. The development of antibiotic resistance is associated with high morbidity and mortality, particularly in the intensive care unit (ICU) setting.

*ESBL* producing isolates (32.78%) were also found common among *VAP Gram negative bacteria* in this study. They are typically plasmid-mediated clavulanate susceptible enzymes that hydrolyze penicillins, expanded-spectrum cephalosporins and aztreonam. Among them (32.78%), 18% isolates were *Enterobacteriaceae* and 14.75% isolates were non-fermenters. Statistically, *Enterobacteriaceae* isolates...
were the commonest ESBL producers. (P<0.01) Among Enterobacteriaceae isolates, 66.66% Escherichia coli isolates, followed by Klebsiella pneumoniae (53.38%) were found to be ESBL producers. This finding was near about similar to Joseph NM et al (Pondicherry, India) study, where Escherichia coli (50%) and Klebsiella pneumoniae (67%) were ESBL producers from VAP cases. However, in Dey et al study, higher frequency 80% of VAP Escherichia coli isolates and 100% of VAP Klebsiella pneumoniae isolates were ESBL producers. In this study, among non-fermenters, Pseudomonas aeruginosa (36.3%) and Acinetobacter spp. (16.66%) isolates were ESBL producers.

Several studies had been carried out at TUTH to determine the prevalence of ESBL among Gram negative isolates. A study conducted by Pokhrel at al in 2004 found 24.27% isolates were ESBL producers and among them 55.0% K. pneumoniae, 50% E coli and 20.69% Pseudomonas spp. were ESBL producers among nosocomial and community LRTI isolates. In Mishra SK et al 2008 study ESBL producing isolates were 77.63% in inpatients and 22.37% in out patients. These results shows that there is significant prevalence of ESBL producing isolates causing LRTI in our hospital.

MBL producing isolates (39%) were found more common than ESBL among VAP isolates. Non-fermenters (92%) were significantly predominant MBL producing bacteria compared to enteric bacilli. (P<0.05). Among non-fermenters, Acinetobacter spp. (66.66%) and Pseudomonas aeruginosa (19%) were MBL producing isolates. Acinetobacter isolates were significantly the commonest MBL producer. (P <0.005). Among enteric bacilli, Klebsiella pneumoniae (33%) isolates were MBL producer. None of E.coli and C. freundii isolates were found to be MBL producer. The MBL producing P. aeruginosain Joseph NM et al study, (Pondicherry, India) was 20%. However, in Dey A et al study, Metallo-β lactamases (MBLs) were produced by 50% of Pseudomonas aeruginosa and 21.74% of Acinetobacter spp. In Nepal, few studies have been done on the prevalence of MBLs, especially from Hospital isolates. In Mishra et al study, MBLs was present in 6 (1.3%) of the total 448 gram-negative isolates. In Shrestha S et al (2010) study at TUTH the prevalence of MBL was 17.43% among nosocomial LRTI, among them Acinetobacter (47.22%), Pseudomonas (23.8%) and of Klebsiellasp (4.17%) were MBL producing isolates. Out of 19 MBL producer, 16 (84.21%) were from ICU.

Apart from drug resistance in Gram-negative bacteria, antibiotic resistance has been observed among Gram-positive isolates too. In this study the incidence of methicillin resistant strains among the Staphylococcus aureus isolates in VAP patient was 75%. Variable findings wasobserved in Trouillet JL et al( 61.5%, France, 1998), Rodrigues et al (40.7%, Brazil,2009),30 Joseph NM (43%, Pondicherry, India,2010), Jones RN (42.5%, North Liberty, Iowa). In a continuous, prospective, multi centre cohort study by Xie DS et al of patients who received MV in 17 ICUs in 17 tertiary care hospitals in Hubei Province, China, of all Staphylococcus aureus isolates, 45.7% were methicillin resistant.

VAP MRSA isolates were commonly resistant to antibiotics such as Clindamycin, fluoroquinolones, trimethoprim–sulfamethoxazole. The very low sensitivity of MRSA strains towards Ciprofloxacin, Cotrimoxazole, Erythromycin is probably due to the indiscriminate empirical use of these drugs. The most effective antibiotics for Staphylococcus aureus along with MRSA was found to be Vancomycin, followed by Doxycycline. Although, the Erythromycin resistance against Staphylococcus aureus isolates was very high (75%) in this study, Erythromycin induced Clindamycin resistant case was not found in all the isolated MRSA strain.

From this study, it becomes clear that resistant bacteria are common in our ICU. It is wise to control this situation before it takes a deadly shape. Following the recommendation given by summit on antimicrobial resistance, unnecessary use of antibiotics, identifying the pathogen, choosing correct antibiotics, limiting excess use of antibiotics, improving resistance surveillance systems will help controlling this situation. Although some resistance is inevitable with the use of antibiotics, steps can be taken to curtail practices that cause and propagate resistance. In this way, we will be able to maintain or prolong the efficacy of existing drug.

The incidence of VAP can be prevented by adopting careful intubation techniques, oral intubation, avoiding gastric over- distension, maintaining adequate endo-tracheal cuff pressure and efficient tracheal toileting.

This study helped us in the early diagnosis of VAP and also to determine the incidence of MDR organisms responsible for VAP. The antibiotic susceptibility pattern helped the clinicians to choose the appropriate antibiotics for prophylactic and treatment purposes.

**Conclusion**

Study shows high prevalence of VAP, MDR along with MRSA or ESBL or MBL producing strains. Thus, suitable control measures must be adopted to cope up this alarming situation with genetic characterization.
Conflict of interest

The authors declare that there is no conflict of interest associated with the study.

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