BACTERIAL ETIOLOGY OF BRONCHOALVEOLAR LAVAGE FLUID IN TERTIARY CARE PATIENTS AND ANTIBIOGRAM OF THE ISOLATES

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ABSTRACT

Bronchoalveolar Lavage (BAL) is a medical technique by which cells and fluids from bronchioles and lung alveoli are withdrawn for diagnosis of disease or evaluation of treatment. Patients with various pulmonary infections follow the procedure of BAL for the disease diagnosis as it has high sensitivity and reliability in diagnosis. To examine the bacterial etiology of BAL fluids among tertiary care patients with pulmonary infections, a cross-sectional study was conducted over a period of three months from August to November 2018. A total of 149 BAL fluid samples were examined and the bacterial agents were isolated and identified by conventional microbiological methods. Out of the 149 samples, 142 samples were culture positive. Among 6 different isolates (4 Gram-negative and 2 Gram-positive), *Pseudomonas aeruginosa* (45 %) was predominant followed by *Klebsiella pneumoniae* (25.3 %). A higher infection rate was seen among males (62.4 %) and in the age group 60-70 years (30.0 %). In addition, 25.4 % (36/149) samples were positive in Acid Fast staining. Bacterial recovery from the BAL fluid was significantly associated with the gender and age of the patients (p<0.05). Gentamycin was the least resisted (1.5 %) by Gram-negative isolates followed by Polymyxin-B (3 %). For Gram-positive isolates, Ofloxacin was the most effective drug resisted by none of the isolates followed by Gentamycin. Among the 157 isolates, 125 (79.6 %) were MDR and 35.0 % of Enterobacteriaceae were ESBL producers. Detection of bacterial agents from BAL fluid can be a basis for successful antimicrobial therapy for patients with pulmonary infections.

Keywords: AFB, Bacterial characterization, BAL, MDR, Pulmonary infection.

INTRODUCTION

Bronchoalveolar lavage (BAL) is a medical procedure in which cells and fluids from bronchioles and lung alveoli are extracted for disease diagnosis (Henderson, 1994). BAL is performed by bronchoscope, the fiber optic wire that is wedged into a bronchus, and sterile saline is pumped in and then withdrawn along with the fluid and cells for analysis (Henderson, 1994). Bronchoalveolar lavage can play a significant role in the diagnosis of various diseases like pulmonary infections, pulmonary malignancy, acute respiratory failure, diffuse infiltrative lung disease, occupational lung disease, pediatric lung disease, post-transplant monitoring of the lung allograft, pediatric lung disease, and others (Meyer, 2007).

In clinical associated pneumonia patients, the treatment failure has been observed which might be linked with the inappropriate choice of the antibiotics, development of superimposed infection due to hospital-acquired pathogens or acquisition of drug-resistant pathogens from the community (Arancibia *et al.*, 2000), and the inconclusive sputum culture report might further mislead the diagnosis (Magazine *et al.,* 2013). The detection of definite etiologic agents is the basis for successful antimicrobial therapy; hence, uncontaminated and appropriate samples for bacteriological diagnosis are of utmost importance. The sample collection using the BAL technique increases the efficacy of the actual pathogen detection by reducing the chances of contamination and is highly convenient for pediatric patients (< 5 years old) as the sputum collection is very difficult in such patients (García-Elorriagaa *et al.*, 2015). Likewise, in immunocompromised patients, the treatment of bacterial pneumonia is still troublesome due to the inabilities of most of the patients to produce sputum (Kahn & Jones, 1987). Therefore, the BAL technique is one of the best options for the proper diagnosis of the disease.

Several microorganisms such as *Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Acinetobacter, Streptococcus pneumoniae, Escherichia coli, Enterobacter, Haemophilus influenzae, Moraxella, Citrobacter, Nocardia,* and many others have been reported from BAL fluid (Magazine *et al.*, 2013). The drug resistance among the clinical (Adhikari *et al.*, 2019; Duwadi *et al.*, 2020; Gyawali *et al.*, 2020; Regmi *et al.*, 2020) and non-clinical isolates (Adhikari *et al.*, 2020; Khadka *et al.*, 2018; Sapkota *et al.*, 2019, 2020) have been widely reported in many studies and bacterial isolates present in the BAL fluid have also been found resistant to the arrays of commonly used antibiotics (Magazine *et al.*, 2013). Bacterial pathogens such as methicillin-resistant *S. aureus* (MRSA), *Acinetobacter baumannii, P.*

aeruginosa, Stenotrophomonas maltophilia, Enterobacter aerogenes, methicillin-resistant *Staphylococcus epidermidis* (MRSE*),* methicillin-resistant *Staphylococcus hemolyticus* (MRSH), *Enterococcus faecium, Burkholderia cepacia* isolated from BAL fluid are found to be MDR (Kim *et al.*, 2012). ESBL-producing bacteria have also been abundantly isolated from both clinical samples (Önnberg *et al.*, 2011; Nasrin *et al.*, 2010) and non-clinical samples (Sapkota *et al.*, 2019).

Furthermore, in developing countries like Nepal, the prevalence of pulmonary diseases like tuberculosis (Banstola, 2012) and pneumonia (Haque, 2019) has created serious problems regarding the high rate of morbidity and mortality. People of all age groups have suffered from those diseases. Due to the lack of proper management and medication, the disease is transmitted from person to person easily. Since the people are not much aware of the threat and possible harms of the disease, they do not follow proper sanitation and medication habits leading to mortality and morbidity. One of the major factors leading to illness is the lack of proper and reliable disease detection techniques. BAL is one of the best innovational techniques discovered which brought a huge breakthrough in the world's medicine. It is a faster, easier, reliable, and sensitive technique in pulmonary disease diagnosis. In light of this discussion, the current study aims to investigate the bacterial spectra of BAL fluid along with the antibiotic resistance patterns of the isolates from the patients attending BPKMCH, Bharatpur.

MATERIALS AND METHODS

Study site and study period

A prospective laboratory-based cross-sectional study was carried out at the Microbiology Laboratory of BP Koirala Memorial Cancer Hospital, Bharatpur, Chitwan. The study was conducted over a period of three months, from August to November 2018.

Sample size, inclusion, and exclusion criteria

A total of 149 BAL fluid samples were collected from the bronchoscopy ward out of the patients suffering from pulmonary infections attending the minor OT Department of BPKMCH for bronchoscopy. The BAL procedures were carried out by the trained medical staff of the hospital. Patients of all age groups and sex were included in the study. Cultures showing mono-microbial growth were included in the study. Samples with improper sealing and labeling were excluded from the study.

Data and specimen collection

Verbal informed consent was sought from each patient before sample collection. They were assured of the fact that the data collected will be used solely for research and their information would be kept confidential. A pre-

structured questionnaire comprising of age, sex, ethnicity, and details regarding bronchoscopy was used to gather the information. Before sample analysis, the BAL fluid was transported to the microbiology laboratory by the registered assistant. The container was first checked whether it was sealed or not or if it was provided with proper labeling with name, age, sex, etc. The sample was properly labeled with the sample number. Then the appropriate samples were considered for further processing without any delay to avoid probable contamination or desiccation.

Sample processing

The sample was processed as soon as it reached the microbiology laboratory following the standard laboratory procedures. The samples were divided into two parts-one was used for preparing smears for direct microscopy while the other was used for culture. Two smears were prepared from each sample for Gram staining and acid-fast staining.

Macroscopic and microscopic examination

The BAL fluids were examined for their appearance, color, consistency, and presence of debris. An even smear of the most purulent part of the specimen was prepared on clean and grease-free slides and subjected to Gram staining and acid-fast staining. The specimen was also observed for any kind of pus cells.

Culture of specimen

The aerobic culture of the specimen was performed under the aseptic condition as soon as the specimen reached the laboratory. The sample was inoculated on MacConkey Agar (MA) and Blood Agar (BA) plates. The plates were aerobically incubated at 37° C for 24 hours. If no growth was observed, then the sample was reported as growth negative. In case of bacterial growth, a single isolated colony was taken and sub-cultured in Nutrient Agar (NA) and incubated at 37° C for 24 h aerobically (Isenberg, 2016).

Isolation and identification of bacteria

The identification of bacterial isolates was done using standard microbiological techniques as described in Bergey's Manual of Systemic Bacteriology which comprised of studying the colonial morphology, physiological tests, staining reactions, and various biochemical tests (Bergey *et al*., 1984). Each organism was obtained in its pure form for performing biochemical and other tests. Gram staining of the isolated culture was performed from the primary culture. Appropriate biochemical tests were performed to identify the organism by its phenotypic characterization. Catalase test, oxidase test, coagulase test, nitrate reduction test, oxidative/fermentative test, triple sugar iron test, SIM test,

MR/VP test, citrate utilization test, urease test were performed as needed (Forbes *et al*., 2007).

Antibiotics susceptibility testing of bacterial isolates

Bacterial susceptibility test to antimicrobial agents was done *in vitro* by modified Kirby-Bauer disc diffusion method, using fresh broth culture of isolates in Mueller Hinton Agar (MHA), following CLSI recommendations. A suspension matching 0.5 McFarland standard (1.5×10^8 cfu/mL) was made in nutrient broth from the pure cultures of bacteria grown overnight on nutrient agar. Mueller-Hinton agar plates were inoculated by the lawn culture method using a sterile cotton swab. Commercially available antibiotic discs ampicillin (10 µg), azithromycin (15 µg), gentamicin (10 µg), ofloxacin (5 µg), polymyxin B (300 µg), co-trimoxazole (25 µg), nitrofurantoin (300 µg), aztreonam (30 µg), imipenem (10 µg), meropenem (10 µg), ceftazidime (30 µg), cefepime (30 µg) were used according to the nature of the organism and different zones of inhibition were measured and the results were interpreted as sensitive, intermediate or resistant by comparing with the standard interpretative table given by Hi Media laboratories. For screening of MRSA, cefoxitin (30 µg) disc was used. Isolates showing an inhibition zone ≤ 21 mm around the cefoxitin disc were considered MRSA (CLSI, 2016). MDR-resistant organisms were differentiated based on their ability to resist three or more classes of antimicrobial agents (Magiorakos *et al*., 2012).

Screening of extended-spectrum beta-lactamase (ESBL) production by phenotypic tests

Ceftazidime and cefotaxime discs were placed on the Mueller Hinton agar plates and incubated at 35° C for 18–24 h. Probable ESBL-producers were the ones showing the zone of inhibition ≤22 mm for ceftazidime (CAZ)(30 µg) and ≤27 mm for cefotaxime (CTX) (30 µg). A phenotypic confirmatory test was done for suspected ESBL producing microorganisms for which antibiotics combinations of ceftazidime + clavulanic acid (CAZ/CAC) (30/10 µg) and cefotaxime + clavulanic acid (CTX/CTC) (30/10 µg) were used (CLSI, 2016). An increase in the zone of inhibition by ≥5 mm around the discs containing cephalosporin with clavulanate over the discs containing cephalosporin alone were ESBL producers (CLSI, 2016).

Statistical analysis

Data analyses were performed using SPSS statistical software ver. 25.0 (SPSS Inc., Chicago, IL, USA). A chi-square test was used to determine the association between pulmonary infection with various attributes such as patients' age, gender, ethnic background, and presence of tuberculosis. The p-value <0.05 was assumed to be statistically significant for the analysis.

RESULTS

Overall growth pattern in samples

Among the 149 samples tested for microbial infection, 142(95.3 %) samples showed at least one bacterial growth. Of those 142 growth positive samples, 127(89.4 %) showed mono-microbial growth and 15(10.6 %) accounted for poly-microbial growth.

Association of bacterial growth with various attributes

Out of the 142 growth positive samples, 92 (64.8 %) were from males and 50 (35.2 %) were from females. There was a strong association between gender and bacterial growth (p<0.05). While the age group 60-70 years accounted for maximum growth 44 (30.9 %) and on the other hand age group, 10-20 years showed the lowest growth rate 3(2.1 %). Age and bacterial growth were significantly associated (p<0.05). Janajati ethnic group was encountered with maximum growth rate with 46 (32.4 %) whereas least growth was accounted from the other ethnic groups 7(4.9 %) but there was no association between ethnic group and bacterial growth. Among the 142 growth positive samples, 36 (24.4 %) were found to be co-infected with *Mycobacterium tuberculosis,* and the remaining 106 (75.6 %) were infected with bacteria other than MTB. But there was no significant association between Tuberculosis and bacterial growth. Among the total samples, 6 (4.1 %) were from those who tested positive with lung cancer (Table 1).

Distribution of isolated bacteria

A total of 157 bacterial growth was observed. Of those, 124 (78.9 %) were Gram negative and 33 (21.1 %) were Gram positive cocci. Among the Gram negative isolates, *Pseudomonas aeruginosa* was the frequently isolated bacterium 64 (51.6 %). *Klebsiella pneumoniae* was the second most frequently isolated bacteria 36 (29.1 %). *Escherichia coli* and *Citrobacter koseri* accounted for 20 (16.1 %) and 4 (3.2 %) respectively. Among 33 Gram positive cocci, *Staphylococcus aureus* accounted for 19 (57.7 %) and *Streptococcus pneumoniae* accounted for 14 (42.3 %), respectively, (Fig. 1).
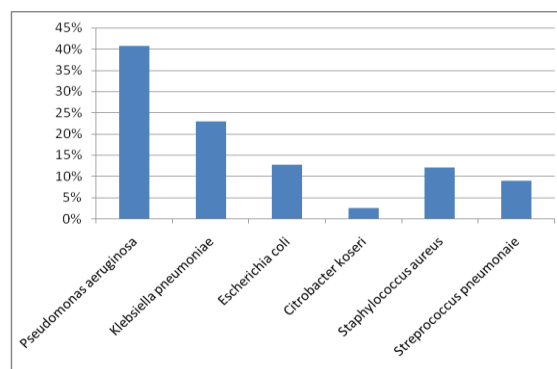

Fig. 1. Distribution of bacterial isolates

Table 1. Association of bacterial growth with various attributes

| Variables	Category	Growth	No Growth	Total	p-Value
Gender	Male	92(98.92%)	1(1.08%)	93	0.007*
	Female	50(89.28%)	6(10.72%)	56	
Age Group	0-10	4(66.67%)	2(33.33%)	6	0.041*
	10-20	3(100%)	0	3	
	20-30	8(88.89%)	1(11.11%)	9	
	30-40	15(100%)	0	15	
	40-50	17(100%)	0	17	
	50-60	26(92.86%)	2(7.14%)	28	
	60-70	44(97.78%)	1(2.22%)	45	
	70-80	25(96.15%)	1(3.85%)	26	
Ethnicity	Brahmin/Chhetri	39(97.50%)	1(2.50%)	40	0.531
	Janajati	46(97.87%)	1(2.13%)	47	
	Madhesi	21(91.30%)	28.70(%)	23	
	Muslim	13(92.86%)	1(7.14%)	14	
	Dalit	16(88.89%)	2(11.11%)	18	
	Others	7(100%)	0	7	
TB infection	Yes	36(100%)	0	36	0.126
	No	106(93.81%)	7(6.19%)	113	

Significant at 5% level of significance

Antibiotic susceptibility pattern (AST)

The panel of 15 antibiotics discs was tested for all the isolates. Ampicillin and carbapenems were the drugs that were effective against all the isolates. Ampicillin was 100 % effective against *P. aeruginosa* and Gram-positive cocci while 92.8 % effective against the Enterobacteriaceae family; whereas carbapenems were 100 % effective against all the isolates. The least effective drug against *P. aeruginosa* was Gentamicin (1.5 %), which was also completely ineffective against the Enterobacteriaceae family and only 12.2 % effective against Gram positive cocci. Amoxyclav, a combination of Amoxycillin and clavulanic acid, was effective against Gram positive cocci and 87.5 % isolates of *P. aeruginosa* but only 54.2 % effective against Enterobacteriaceae isolates (Table 2).

Distribution of MDR and MRSA isolates

Out of the total 157 isolates, 125 (79.6 %) isolates were MDR whereas the remaining 32 (20.4 %) isolates were non-MDR. Among the 19 *S. aureus* isolates, 10 (52.6%) were MRSA. *P. aeruginosa* dominated the MDR count accounting for 48.8 % of total isolates. Following it was *K. pneumoniae* with 23.2 % of isolates being MDR. The least MDR isolates were accounted for from *C. koseri* (0.8

%). Among Gram positive cocci, *S. aureus* accounted for 7.2 % isolates and *S. pneumoniae* accounted for 4 % among total MDR isolates (Fig. 2).
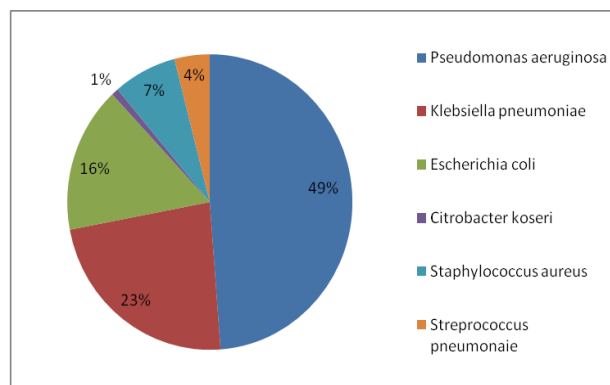
Fig. 2. Distribution of MDR isolates

Frequency of ESBL-producing Enterobacteriaceae

Among 60 enterobacteriaceae isolates, 31 (51.7 %) isolates showed ESBL screening test positive. On performing the confirmatory test, 11 (18.3 %) isolates of *K. pneumoniae,* 8 (13.3 %) isolates of *E. coli,* and 2 (3.3 %) of *C. koseri* were found to be ESBL producers, as shown in (Table 3).

Table 2. Antibiotic Susceptibility Pattern of the isolates

Antibiotics	*P. aeruginosa*			Enterobacteriaceae			Gram positive Cocci		
	S	I	R	S	I	R	S	I	R
Ampicillin	100.0%	0.0%	0.0%	92.8%	7.2%	0.0%	100.0%	0.0%	0.0%
Amoxyclav	87.5%	9.3%	3.1%	54.2%	30.0%	15.8%	100.0%	0.0%	0.0%
Azithromycin	71.8%	25.0%	3.1%	21.4%	48.5%	30.1%	63.6%	12.2%	24.2%
Cefoxitin	92.1%	7.8%	0.0%	27.1%	55.7%	17.2%	63.6%	36.4%	0.0%
Gentamicin	1.5%	26.5%	71.8%	0.0%	31.4%	68.6%	12.2%	0.0%	87.8%
Ofloxacin	6.2%	23.4%	70.3%	10.0%	20.0%	70.0%	0.0%	63.6%	36.4%
Polymyxin B	6.2%	32.8%	60.9%	2.8%	44.2%	53.0%	24.2%	24.2%	51.6%
Co-trimoxazole	85.9%	14.1%	0.0%	78.5%	12.8%	8.7%	63.6%	0.0%	36.4%
Nitrofurantoin	57.8%	12.5%	29.7%	36.7%	5.0%	58.3%	54.5%	36.4%	9.1%
Aztreonam	39.1%	15.6%	45.3%	46.7%	5.0%	48.3%	63.6%	15.2%	21.2%
Imipenem	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%
Meropenem	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%	100.0%	0.0%	0.0%
Ceftazidime	43.8%	20.3%	32.8%	55.0%	3.3%	41.7%	39.4%	9.1%	51.5%
Cefepime	23.4%	7.8%	68.8%	16.7%	25.0%	58.3%	39.4%	15.2%	45.5%

S= Sensitive, I= Intermediate, R= Resistant

Table 3. Frequency of ESBL-producing isolates

Organism	Significant growth	Screening test positive	Confirmatory test positive (combination disk method)
K. pneumoniae	36	17	11(30.6%)
E coli	20	12	8(40.0%)
C. koseri	4	2	2(50.0%)
Total	60	31	21(35.0%)

DISCUSSION

The diagnosis of respiratory tract diseases can be aided by bronchoscopy which is one of the best and frequently used techniques. By performing bronchoscopy, Bronchoalveolar lavage (BAL) fluid can be obtained for the disease diagnosis. BAL fluid is more sensitive and ensures for disease diagnosis. Besides this, bronchoscopy can be carried out in over-aged patients (Radha *et al*., 2014). The sample obtained using the BAL technique increases the efficacy of the actual pathogen detection by reducing the chances of contamination.

A total of 149 BAL fluid samples were collected and processed of which growth was seen in (95.3 %) of the samples. A study of diagnostic utility of bronchoalveolar lavage conducted in India found 88% of BAL fluid samples were growth positive (Radha *et al*., 2014). The study regarding the diagnostic utility of BAL with malignancies was performed by Hummel *et al*. (2008), which reported only 47 % of the sample gave growth positive results. This high degree of variation depends highly upon the conditions in which patients are being treated with antimicrobial agents and also upon the cultural techniques. In this study, a total of 142 isolates were observed among which 94.4 % were Gram-negative organisms, whereas 5.6 % were Gram-positive organisms. Among 134 Gram-negative isolates, *Pseudomonas aeruginosa* (51.6 %) was the most prevalent isolate. However, a different result was obtained in a study where *Klebsiella pneumoniae* (39.4 %) was the main isolate (Radha *et al*., 2014). Moreover, a study of the microbiology of bronchoalveolar lavage in infants reported *Moraxella* sp. and *Streptococcus mitis* as the major isolates (Palma-alaniz & Ruelas-vargas, 2015). A high prevalence of *P. aeruginosa* in cases of pulmonary infection is due to its easy distribution in the air via any environmental sources like soil and water. Along with this, the transmission of bacteria is aided by the cough aerosols of other infectious patients (Woo *et al*., 2018; Thapa *et al*., 2016).

Out of the 142 growth positive samples, 64.8% were from males and 35.2% were from females. There was a strong association between gender and bacterial growth (p<0.05). The higher occurrence of infection in a male could be because of the diverse exposure of males on various fields than females. All of the age groups after 30 years showed a higher rate of infectivity, including a 100% growth rate among the age group 30-40 and 40-50 years (p<0.05).

This may be due to higher exposure in an environment which increases the risk of infection and also the weakening of immunity in the host during old age and this age group are more likely to suffer from pulmonary infections more due to the food habits like smoking and other malfunctions of the body like diabetes and blood pressure alterations (Blot *et al*., 2014). Janajati encountered a maximum percentage of the bacterial incidence than other ethnic groups followed by Brahmin/Chhetri (p>0.05).

The high proportion of infection from the ethnic group can be attributed to the culture and tradition of these people along with the food and sanitary habits of the particular people. Among 149 samples, 25.4 % showed AFB positive (p>0.05). This was in tune with a study with 24 % AFB-positive samples (Radha *et al*., 2014). The proximity of bacteria with tuberculosis patients may be due to the weakened host immunity which help the successful colonization of the bacteria (Flynn, 2004). In various researches, it has been observed that there is a significant relationship between previous TB and lung cancer (Keikha & Esfahani, 2018).

Antibiotic susceptibility testing of the isolated bacteria revealed that gentamicin, a commonly prescribed antibiotic, was resisted by many isolates. This was contrary to the research conducted in Poland (Semczuk *et al*., 2015). In terms of Gram positive bacteria, isolates were 100 % sensitive to ampicillin whereas the majority of the isolates were resistant to gentamycin and polymyxin-B. In a study in Uganda, ampicillin was the least effective drug (82 %) (Kitara *et al*., 2011). In this study, out of the total 157 isolates, 79.6 % isolates were MDR. This was in tune with the study which showed a majority of the isolated bacteria were resistant to antibiotics (Semczuk *et al*., 2015).

The occurrence of MDR isolates could be due to the evolving nature such as, simple nutritional requirement and ability to thrive in a wide range of temperature which assists gain resistance over many traditional and commonly used antibiotics. The problem of antibiotic resistance has been increasing rapidly and it has become a serious burden in the medical world. In addition, among 60 Enterobacteriaceae isolates, 35.0 % were ESBL producers. The emergence of ESBL is mainly attributed to a resistant gene carried on an epidemic plasmid that can dessiminate and exist in the environment (Aibinu *et al*., 2007).

CONCLUSION

The current study reveals a high bacterial prevalence in the BAL fluid samples from the patients with pulmonary infections with the higher recovery of bacterial isolates in males and the age group 60-70 years. Out of the total 157 isolates, 79.6 % were MDR. In addition, among 60

Enterobacteriaceae isolates, 35.0 % were ESBL producers. The emergence of multidrug-resistant bacteria, some of which being ESBL-producers, in BAL samples is worrisome and concerned authorities should consider this to abate their incidence and dissemination.

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