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Original Article

Microbiology Section

Diagnostic Utility of Real Time Multiplex PCR for Identification of Atypical Bacterial Respiratory Pathogens

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ABSTRACT

Introduction: Infections are common in cirrhosis and is the leading cause of progression of liver failure and increased mortality. Non-specific findings on radiology prompt a need for a diagnostic armamentarium targeting both typical as well as atypical pathogens. With the advent of molecular diagnostic techniques, an early diagnosis and timely initiation of therapy prevents the injudicious use of antibiotics.

Aim: To determine the utility of real time multiplex Polymerase Chain Reaction (PCR) as a diagnostic aid in identification of atypical bacterial respiratory pathogens in cirrhotic patients.

Materials and Methods: The observational study was conducted at Tertiary Hepatobiliary Centre, Institute of Liver and Biliary Sciences Hospital, New Delhi, India, from April to September 2018. Bronchoalveolar Lavage (BAL)/mini-BAL samples were collected from 88 suspected cases of pneumonia for routine laboratory work-up as per clinician request. The lower respiratory tract samples were subjected to semi-quantitative culture and PCR. Results were analysed on the basis of aetiological categorisation of liver diseases, radiological and microbiological findings.

Results: Among 88 samples, nine were positive either by culture or PCR. PCR analysis revealed three positive samples. A sample positive for *Legionella* species on PCR showed growth of *A. flavus* in culture. Another sample was positive for both *Legionella* species and *S. aureus* on PCR. The third sample was positive for *S. aureus* on PCR and on culture grew Methicillin Sensitive *S. aureus* (MSSA).

Conclusion: Liver disease is a risk factor for development of *Legionella* and other atypical pathogens. Therefore, it becomes important to actively look for such pathogens during the work-up of a patient with clinical suspicion of pneumonia. Thus, early diagnosis help in limiting the use of indiscriminate antimicrobials, and timely initiation of targeted antimicrobials. Use of molecular techniques like PCR help in identifying the atypical pathogens which are neglected and thus determines the course of treatment.

Keywords: Antibiotic management, Cirrhosis, *Legionella*, Pneumonia

INTRODUCTION

In Chronic Liver Disease (CLD), a complex interplay of translocation of gut microbes, overt stimulation of immune cells, complement system deficiencies and impaired neutrophil function predisposes a patient to bacterial and fungal infections [1]. Cirrhosis associated immune dysfunction syndrome, an entity increases the susceptibility of development of spontaneous bacterial infections, community-acquired and hospital-acquired infections by both typical and atypical pathogens. Spontaneous Bacterial Peritonitis (SBP), urinary tract infection, pneumonia and bacteraemia are most prevalent infections in liver disease patients [2]. Ascites and lungs are common sites of bacterial infections. Cirrhosis predisposed infections have a poorer outcome, increasing the mortality four fold. Studies reports that more than 30% cases of Acute on Chronic Liver Failure (ACLF) are triggered by bacterial infections and 30-50% of deaths in cirrhotic patients are due to infections [2,3].

Community acquired pneumonia an infectious disease can be responsible for high morbidity and mortality when treated inappropriately. Common bacterial pathogens include *Streptococcus pneumoniae* and *H. influenzae*. Other fastidious pathogens include *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumophila* that accounts for 15% of total cases [2]. These pathogens serves as a causative agent for atypical pneumonia and do not respond to the β-lactam drugs, thus accentuating the need for a proper detection system. Conventional methods such as culture and serology analysis are time-consuming with low sensitivity and specificity, and therefore needs to be supplemented with more accurate tests. With the advent of multiplex PCR, early identification of multiple pathogens is now possible. Thus, this study was conducted to assess the role of PCR as a diagnostic aid in identifying the atypical organisms that are difficult to culture. Due to the paucity of literature in liver disease patients, this study was carried out to study the atypical pathogens as an aetiologic cause in pneumonia.

MATERIALS AND METHODS

In the present observational study, a total of 88 cases suspected with pneumonia, admitted to Tertiary Hepatobiliary Centre, Institute of Liver and Biliary Sciences Hospital, New Delhi, India, from April to September 2018 were included. Ethical clearance was taken from the Institute Ethics Committee (IEC/2018/61/MA15) before commencement of the study. Cirrhotic patients with features suggestive of pneumonia were selected. Patients with: (i) Disseminated Intravascular coagulation/extremely moribund individuals with expected survival <48 hours; (ii) Inappropriately collected samples which fall under rejection criteria; (iii) Smear positive/culture positive/Cartridge Based Nucleic Acid Amplification Tests (CBNAAT) positive samples for *M. tuberculosis* were excluded from the study. After obtaining an informed written consent, BAL/mini-BAL samples were collected from all the participants for routine laboratory work-up as per clinician request.

Both conventional culture media and multiplex real time PCR were used to detect bacterial pathogens. Culture media for bacterial growth included Blood and MacConkey agar [4]. The inoculated mediums were incubated for 24-48 hours in a 5% CO_2 incubator. For fungal examination, all the samples were inoculated on SDA media and incubated for four weeks [4]. For molecular analysis, DNA was extracted using QIAamp DNA mini kit (Qiagen, Germany). Multiplex PCR was carried out using real time thermocycler (Rotor-Gene Q, Qiagen) and standard detection kit (FTD Bacterial

pneumonia_CAP, Fast Track Diagnostics, Luxemburg, catalogue no. FTD-29.1-32). Organisms targeted by multiplex real time PCR included *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Legionella pneumophila/longbeachae*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*. Results were analysed on the basis of aetiological categorisation of liver diseases, radiological and microbiological results.

RESULTS

In the present study, samples from 69 men and 19 women aged 2-71 years were analysed. The diagnosis of patients included CLD (n=41), ACLF (n=26), Acute Liver Failure (ALF) (n=11), Non Cirrhotic Portal Fibrosis (NCPF) (n=1), and other condition: Pancreatitis, Secondary HLH, Kidney transplant, Carcinoma, Biliary atresia, Visceral Leishmaniasis (n=11). The aetiologies of the liver disease have been summarised in [Table/Fig-1]. All these patients with clinical suspicion of pneumonia underwent radiological examination, the findings of which have been categorised in [Table/Fig-2]. In the microbiology laboratory, a total of 9 out of 88 cases were positive either by culture or PCR [Table/Fig-3]. Culture results showed growth of *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Aspergillus flavus* and *Aspergillus fumigatus*. Three samples were positive by PCR, among them one sample was positive for *Legionella* species on PCR and on culture this sample showed growth of *A. flavus*. The second sample was positive for both *Legionella* species and *S. aureus* on PCR. The third sample was positive for *S. aureus* on PCR and grew MSSA on culture.

Aetiology	Percentage n (%)
Alcohol	46 (52.2%)
Nonalcoholic steatohepatitis (NASH)	14 (15.9%)
Viral	11 (12.5%)
Autoimmune	3 (3.4%)
Cryptogenic	4 (4.5%)
Drug induced	2 (2.3%)
Others	8 (9.1%)

[Table/Fig-1]: Aetiologies of liver diseases among cases (n=88) under study.

Radiologic presentations	Frequency n (%)
Normal	6 (6.8%)
Consolidation, opacities	49 (55.7%)
Pleural effusion	24 (27.3%)
Pleural effusion and consolidation	5 (5.7%)
Pneumonitis	3 (3.4%)
Consolidation and Alveolar haemorrhage	1 (1.1%)

[Table/Fig-2]: Radiologic findings of the cases (n=88) under study.

Cases	PCR	Bacterial culture	Fungal culture	Microbial sp.
1	+		+	*Legionella* (P), *A.flavus* (C)
2	+			*Legionella* (P), *S.aureus* (P)
3	+	+		*S.aureus* (P,C)
4		+		*K. pneumoniae* (C)
5		+		*K. pneumoniae* (C)
6		+	+	*K. pneumoniae* (C), *A. baumannii* (C)
7			+	*A.flavus* (C)
8		+	+	*A.flavus* (C), *K. pneumoniae* (C),
9			+	*A. fumigatus* (C)
Total	3	5	5	

[Table/Fig-3]: Microbial aetiological agents in the cases (n=88) under study.
P- Positive for PCR; C- Positive for culture

DISCUSSION

Infections in CLD patients trigger rapid deterioration and liver disease progression. In 25% to 47% of hospitalised cirrhosis patients, the precipitating factor leading to acute decompensation are bacterial infections [5]. The condition of most of these patients rapidly deteriorate, ultimately progressing to multiple organ failure and septic shock. Pneumonia is a major cause of morbidity and mortality in cirrhotic patients [5]. Chest radiography is usually the first examination to be obtained for the evaluation of a patient with a suspicion of pneumonia. An overlapping pattern caused by atypical pathogens makes it essential to have a complete diagnostic armamentarium targeting typical and atypical pathogens [6]. Atypical pathogens are fastidious organisms that are difficult to culture. In our study, in addition to conventional culture methods, the respiratory panel was used to target the atypical pathogens that helped us to broaden the targeted organism for diagnosis and understand the respiratory pathogen profile in CLD patients.

PCR based nucleic acid detection methods have been proven to be more sensitive as compared to culture and serology testing. In a study by Pinar A et al., the multiplex methods used for diagnosis could identify 41.4% of cases as opposed to 23.4% using conventional methods and the most prevalent pathogens included *S. pneumoniae*, *H. influenzae* and *M. pneumoniae* [7]. In another study by Aydemir O et al., on BAL samples, reported better diagnostic value of multiplex PCR with 63.5% cases identification in comparison to culture which only yielded 31.5% of result positivity [8].

Due to limited number of studies on infections in liver diseases, the spectrum of respiratory pathogens in pneumonia patients is not well documented. Some of the most prevalent bacterial pathogens in liver disease patients with pneumonia include *Enterococci, S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, *Legionella*, Enterobacteriaceae, *P. aeruginosa* and *S. aureus* [5]. In our study, the aetiological agents were *Legionella* (detected by PCR), *S. aureus* (detected by both PCR and culture), Enterobacteriaceae and *Acinetobacter baumannii* (detected by culture) and *A. flavus* and *A. fumigatus* (detected by culture). In addition, co-infections of *Legionella* infections with MSSA and *A. flavus* were also observed. However, unlike previous studies, the common respiratory pathogens such as *S. pneumoniae*, *H. influenzae* and *M. pneumoniae* were detected in our study population.

In our study, the two positive cases of *Legionella* species helped in identification of patients with atypical pathogens and early initiation of target therapy. The data on Legionellosis in liver disease from India is limited. Till date, no cases of *Legionella* in a cirrhotic patient have been reported. The bacterium *Legionella* has a unique susceptibility profile to fluoroquinolones and macrolides only. Non-specific clinical and radiological presentation makes it important to identify patients at risk of *Legionella* infection. Risk factors associated with *Legionella* infection includes people with age 50 years or older, smokers, COPD patients or immunosuppressed individuals, with underlying co-morbid conditions such as diabetes, renal failure or liver failure [9]. Thus, our study emphasises the need to test cirrhotic patients for *Legionella* infection. With more than 15 serotypes, *L. pneumophila* require special culture media like Buffered charcoal yeast extract agar, that requires 4-10 days to grow and this method is incapable of detecting 'Viable But Not Culturable' (VBNC) cells [10]. *Legionella* antigen testing, a rapid diagnostic test for *L. pneumophila* are used on urine samples but they can detect only *L. pneumophila* (serotype 01) [11]. Thus, PCR has an added advantage of detecting *L. pneumophila* non1 serotype, as in immunocompromised population non *L. pneumophila* is more prevalent [12]. Qin T et al., reported four cases of cirrhosis with Legionnaire's disease from China where all the four cases were diagnosed using PCR and were successfully treated [10].

Fungal pathogens also pose a similar threat to the CLD patients [13]. Prolonged hospital stay, cirrhosis and antimicrobial therapy often leads to emergence of fungal infections in liver disease patients. In our study, 4 out of 88 cases had positive culture for *Aspergillus* species including both *A. fumigatus* and *A. flavus*. Liver diseases serve as a risk factor for invasive aspergillosis [14]. Differentiation between colonisation and Invasive Pulmonary Aspergillosis (IPA) mandates a complete clinical and radiological evaluation. IPA is a fatal cirrhosis associated complication with mortality rate exceeding 50% because of the immunosuppression in End Stage Liver Disease [ESLD] [14]. In a study by Prattes J et al., prevalence rate of IPA was found to be 1.3% [14]. In another study, fungal colonisation was found to be 25% in ESLD patients of which Aspergillosis constituted upto 3% [15]. Increased prevalence of IPA or aspergillus colonisation has been observed in the past decade, which mandates the need to screen fungal infections in liver disease patients.

LIMITATION

In the present study, the sample size was relatively small. Further studies on larger study cohort are required to validate these findings. In addition, while multiplex PCR was used for all the samples, culture and serology for *Legionella*, *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* could not be done.

CONCLUSION

Liver disease patients need to be treated as a separate group of patients due to their predisposition to infections. An immunosuppressive state mandates the judicious use of antimicrobial prophylaxis to avoid development of antimicrobial resistance. Use of molecular techniques like PCR helps in identifying the atypical pathogens which is often neglected. Our study indicates that *Legionella* infection is a risk factor in liver disease patients. With a different susceptibility profile including macrolides and fluoroquinolones, early diagnosis help in limiting the use of indiscriminate antimicrobials, and timely initiation of targeted antimicrobial treatments.

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