Original Article

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Comparison of Molecular Method and Culture in Detection of Community Acquired Pneumonia in Under Five Year Children in Assam, India

Microbiology Section

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ABSTRACT

Introduction: Pneumonia is the single largest infectious cause of death in children worldwide. Aetiology of pneumonia can be identified using multiple diagnostic tools including culture, serology and Polymerase Chain Reaction (PCR); common pathogens include *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, some atypical bacteria like *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*.

Aim: To find out the bacteriological agents causing Community Acquired Pneumonia (CAP) in under five year children and to compare the conventional culture and PCR in identifying the pathogen.

Materials and Methods: This cross-sectional study was undertaken in the Department of Microbiology and Department of Paediatrics in a tertiary care centre of Assam, India, between March 2016 to September 2018. The study was undertaken with 200 under five year old children who were clinically diagnosed as CAP. Oropharyngeal (OP) swabs and blood culture were processed for bacteriological culture. PCR assay of OP swabs for *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* etc., including atypical bacteria like *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* were performed. Data entry, database management and statistical analysis were performed using Epi-Info software version 7.0.

Results: A total of 200 subjects were included in the present study with a male:female ratio of 1.63:1. Most of the study subjects were <11 months of age. Most common isolates were *Streptococcus pneumoniae* (26.5%) and *Staphylococcus aureus* (25%) from OP swabs; blood culture revealed mostly *Staphylococcus aureus* (59%) and *Streptococcus pneumoniae* (25%). PCR assay of OP swabs were found positive mostly for *Staphylococcus aureus* (47%), *Streptococcus pneumoniae* (21.5%) while two children were positive for *Haemophilus influenzae*; *Bordetella pertussis* was detected in one child. On evaluation, PCR assay in detecting the bacterial pathogen was found statistically more significant than conventional culture of OP swabs ($p<0.05$).

Conclusion: *Staphylococcus aureus* and *Streptococcus pneumoniae* were the most common bacterial organisms in the study. PCR assay was found to be more useful in diagnosing the pathogen for bacterial pneumonia including those difficult to grow in conventional culture.

Keywords: Atypical bacteria, Bacteriological culture, Polymerase chain reaction, Selective medium

INTRODUCTION

The CAP is one of the leading causes of death in children under five years of age in developing countries like India. Failure to diagnose promptly and accurately may lead to increased mortality among them. Pneumonia accounts for 15% of all deaths of children under five years, killing 8,08,694 children in 2017 [1]. Varied clinical presentation of paediatric CAP at different stages of illness like fever, cough, dyspnea, wheeze, chest or abdominal pain, lethargy, vomiting etc., or signs of sepsis, tachypnea, tachycardia, hypoxia, respiratory distress lead to diagnostic complexity in clinical setting. CAP can be caused by many different microorganisms, mostly by viruses and bacteria. Inability to distinguish the aetiological agents based on the clinical presentation has impelled the diagnostic laboratories to adopt advanced diagnostic modalities including PCR in recent times. Common difficulties encountered in studying the aetiology of CAP in children are low yield of blood cultures, lack of satisfactory sputum specimens difficulty to obtain bronchial aspirate and Bronchoalveolar Lavage (BAL) etc., [2]. The published literature worldwide revealed that viruses are the main cause of pneumonia in children less than two years of age [3,4]. Besides respiratory viruses, bacteria like *Streptococcus pneumoniae* and *Haemophilus influenzae* type B are common cause of pneumonia in children aged two to five years [3]. *Streptococcus pneumoniae* remains the most common bacterial cause of CAP; though the widespread use of pneumococcal immunisation has reduced the incidence of invasive disease [5]. The other common pathogens include *Haemophilus influenzae* (including non typeable strains), and *Staphylococcus aureus*. Atypical agents include *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Legionella pneumophila* [6]. In order to aid in formulating better therapeutic options or empirical antimicrobials and prophylactic measures like development of vaccine, accurate and prompt identification of aetiological agents of CAP is a must.

As there is a paucity of the published literature in North East India region about CAP in children with a comparative evaluation of the diagnostic modalities [7,8]. The present study was undertaken to determine the bacterial aetiology of CAP in children younger than five years using bacterial culture and PCR and to compare both the methods.

MATERIALS AND METHODS

This hospital-based cross-sectional study was undertaken between March 2016 to September 2018 with 200 study children below five years of age, clinically diagnosed as CAP who were admitted in the Paediatric ward of Assam Medical College and Hospital, Dibrugarh, Assam, India, during that period. The study was approved by the Institutional Ethics Committee of the institute (Reg No. ECR/636/Inst/AS/2014, dated: 10/3/15). Informed written consent was obtained from the parents or the legal guardians of the study subjects.

Inclusion criteria: Subjects with clinical symptoms and signs of pneumonia fever, cough, tachypnea according to World Health Organisation guidelines [9], and/or findings of crackles, bronchial breathing or diminished breathe sounds on auscultation and those with presence of consolidation on chest X-ray were included in the study.

Exclusion criteria: All cases of pneumonia on antibiotics prior to sample collection and those with suspected hospital acquired pneumonia were excluded from the study.

Sample size calculation: Sample size was calculated using Raosoft online software tool considering prevalence of 16% as reported in a recent study from this region [7].

Cases were categorised into 'pneumonia' and 'severe pneumonia' according to WHO criteria [9]. Socio-economic status was measured using modified Kuppuswamy scale [10].

Culture Methods

Oropharyngeal swabs in duplicate were collected from all the participants using sterile flocked nylon swabs and transported to the microbiology laboratory for immediate processing as per standard protocol [11]. The sample placed in collection vial was used for culture and the other swab placed in transport media (phosphate buffered saline) was vortexed and divided into three aliquots, one used for PCR analysis, the second aliquot used for gram staining was centrifuged at 1,500xg for 15 minutes. The supernatant was decanted, and the smear was made from the deposit. OP swab culture plating was performed on 5% sheep blood agar (in house prepared sheep blood), MacConkey agar, chocolate agar, Levinthal's medium (selective medium for *Haemophilus influenzae*), Pleuropneumonia-like Organism (PPLO) medium (selective medium for *Mycoplasma pneumoniae*) and Buffered Charcoal Yeast Extract (BCYE) medium (selective medium for *Legionella pneumophila*). All dehydrated culture media procured from HiMedia labs, Mumbai, India. Inoculated blood agar media and chocolate agar media were incubated in an atmosphere of 5-10% CO_2. The BCYE and PPLO media were incubated for three to five days and one to three weeks, respectively.

Two blood samples were taken from two venipuncture sites and collected in two blood culture bottles containing Trypticase soy broth (HiMedia Labs, Mumbai, India). The blood culture bottles were incubated aerobically at 37°C and subculture done at least once during the first day and every second day till seventh day with a final subculture then. Identification and speciation of the culture isolates was performed as per standard microbiological techniques [10,11].

Polymerase Chain Reaction Assay

Deoxyribonucleic Acid (DNA) extracted from the OP swab sample using QiAmp DNA mini kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Characterisation of the different bacterial pathogens was done by performing PCR targeting specific genes for these bacterial pathogens [12-14]. Primer sequences used for the detection of the bacterial pathogen listed in [Table/Fig-1].

Amplification for the detection of *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* and *Klebsiella pneumoniae* was started with an initial denaturation at 95°C for two minutes, followed by 40 cycles of PCR at 95°C for 15 seconds, 50°C for 30 seconds and 75°C for 30 seconds successively; followed by final extension at 72°C for five minutes. The PCR condition for the detection of *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Bordetella pertussis* and *Legionella pneumophilia* was 40 cycles of PCR at 95°C for 30 seconds, 64°C for 40 seconds and 72°C for 20 seconds successively, initial denaturation and final extension being the same.

The PCR protocol for *Haemophilus influenzae* includes another round of PCR for the detection of *Haemophilus influenzae* type B with initial denaturation at 95°C for two minutes, followed by 25 cycles of PCR at 94°C for one minute, 60°C for one minute and 72°C for one minute successively; followed by final extension at 72°C for 10 minutes. PCR assays were carried out in the thermal cycler (Thermo Fisher Scientific, Inc., Waltham, MA). The PCR end products were analysed by electrophoresis on 1.5% agarose gel stained with ethidium bromide and the bands were visualised under gel documentation system, Bio-Rad. In house control DNA previously verified by sequence analysis was used as positive control for difficult to culture organism. Cultured stocks of following American Type Culture Collection (ATCC) strains were used as controls namely, *Streptococcus pneumoniae* ATCC 49619, *Haemophilus influenzae* ATCC 49766, *Klebsiella pneumoniae* ATCC 700603, *Staphylococcus aureus* ATCC 25923 (all procured from Microbiologics, USA).

STATISTICAL ANALYSIS

Data entry, database management and statistical analysis were performed using Epi-Info software version 7.0. Chi-square test was used for calculating p-value and p-value <0.05 was considered to be statistically significant.

RESULTS

During the study period a total of 200 subjects were enrolled. Male:female ratio was 1.63:1 and most study subjects were <11 months of age 147 (73.5%). Majority of them were rural inhabitant 160 (80%) and from middle class families 99 (49.5%). Mean (±SD) age was 10.18±11.71 months, mean (±SD) height 76.99±19.83 cm and mean (±SD) body weight 6.75±4.37 Kg. Mean duration of illness was found to be 4.47 days in this study. Symptoms like fever, cough and breathlessness were present in

Organism	Target gene	Primer	Sequence (5' to 3')	Amplicon size (bp)
Streptococcus pneumoniae	Lyt A	FPRP	CAACCGTACAGAATGAAGCGG TTATTCGTGCAATACTCGTGCG	319
Haemophilus influenzae	16sRNA	FPRP	TTGACATCCTAAGAAGAGCTC TCTCCTTTGAGTTCCCGACCG	167
Staphylococcus aureus	16sRNA	FPRP	AGAGTTTGATCATGGCTCAG GGACTACCAGGGTATCTAAT	798
Klebsiella pneumoniae	16s-23S ITS	FPRP	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130
Bordetella pertussis	IS481	FPRP	CCCATAAGCATGCCCGATTGAC CGCACAGTCGGCGCGGTGAC	121
Mycoplasma pneumoniae	Cytadhesin P1 gene	FPRP	GACCATTCCACCCAGCCCCCAGC GTTCAGCGAGTGGGGTGCGTACAATA	343
Chlamydophila pneumoniae	Outer membrane protein A (omp1)	FPRP	TGCGCTACTTGGTGCGACGCTA CGCCTTTATAGCCCTTGGGTTTRTT T	571
Legionella pneumophila	Macrophage infectivity potentiator (mip)	FPRP	CGCTCAATTGGCTTTAACCGAACA G CGCTRCGTGGRCCATATGCARGAC	450
Haemophilus influenzae type B	bexA	FPRP	GCGAAAGTGAACTCTTATCTCTC GCTTACGCTTCTATCTCGGTGAA	480

[Table/Fig-1]: Aetiological agents with target genes and primer sequences for PCR assay.

all the children (n=200); wheeze was found in 196 (98%) of them [Table/Fig-2]. Most of the subjects (n=176) were categorised as "Pneumonia" while 24 number of children were found to be of severe pneumonia category.

Study groups		n=200	Percentage (%)
Age group (months)	0-12	147	73.50
	>12	53	26.50
Sex	Female	76	38.00
	Male	124	62.00
Habitat	Rural	160	80.00
	Urban	40	20.00
Socio-economic status	Lower middle class and above	99	49.50
	Upper lower class	60	30.00
	Poor	41	20.50
Mean±SD age (months)		10.18±11.71	
Mean±SD height (cm)		76.99±19.83	
Mean±SD weight (kg)		6.75±4.37	

[Table/Fig-2]: Demographic details of study groups.

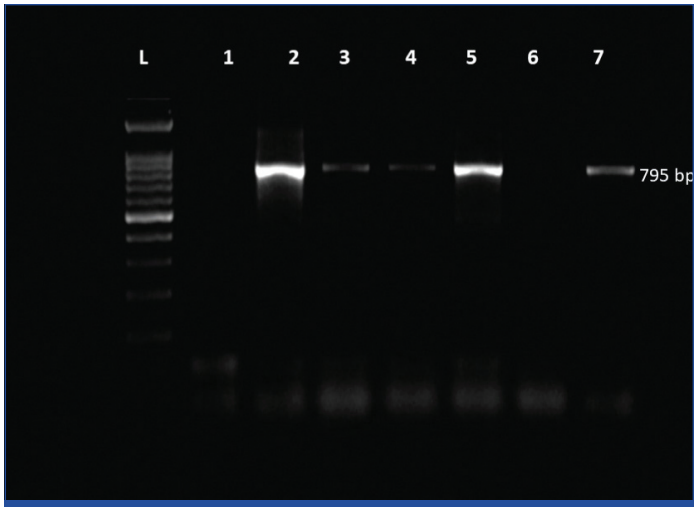
Bacterial aetiology by culture method: Out of 200 OP swabs samples processed for bacteriological culture, total of 128 isolates were recovered from 121 study subjects (isolation rate 64%); 79 cases revealed normal flora of throat and were considered culture negative. Out of 128 bacterial isolates *Streptococcus pneumoniae* 34 (26.5%)and *Staphylococcus aureus* 32 (25%) almost equally leading the tally followed by *Klebsiella pneumoniae* 22 (17.2%). Organisms isolated are shown in [Table/Fig-3]. The isolation rate of aerobic blood culture was 27 (13.5%), organism isolated predominantly *Staphylococcus aureus* 16 (59%) followed by *Streptococcus pneumoniae* 7 (25%).

Organisms	Culture of OP swab	Blood culture
Streptococcus pneumoniae	34	7
Klebsiella pneumoniae	22	0
Staphylococcus aureus	32	16
Escherichia coli	12	0
Acinetobacter species	2	1
Aeromonas hydrophila	1	0
Pseudomonas aeruginosa	3	0
Enterococcus species	6	0
Serratia marcescence	0	1
Proteus mirabilis	1	1
Burkholderia species	1	1
Yeast	14	0
Total	128	27

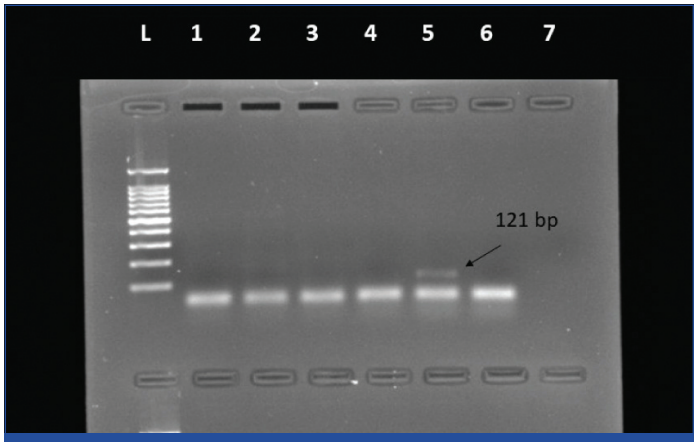
[Table/Fig-3]: Organisms isolated (Oropharyngeal swab culture and blood culture).

Bacterial aetiology by PCR assay: Out of 200 samples tested 152 (76%) were found positive for bacterial pathogen by PCR assay [Table/Fig-4,5]. Total *Staphylococcus aureus* isolates including co-infected were 94 (47%) followed by *Streptococcus pneumoniae* 43 (21.5%) [Table/Fig-6,7].

In this study, OP swab culture was positive in 121 (60.5%) of cases while PCR was positive in 152 (76%) of them ($p<0.05$). It was found that the PCR assay in detecting the bacterial pathogen was statistically more significant than conventional culture of OP swabs [Table/Fig-8]. When analysed the PCR assay was found to be 72.3% sensitive (95% CI 65.8-78.3%) and 64% specific (95% CI 52.0-74.7%) in diagnosing bacterial pneumonia. Factors like age, sex, habitat, socio-economic status were not found to be statistically significant [Table/Fig-9].



[Table/Fig-4]: Gel image showing positive band (798 bp) for *Staphylococcus aureus*. 100 bp ladder (L); Lane 1: Negative control; Lane 2: Positive control; L3,4,5,7: Positive sample; Lane 6: Negative sample



[Table/Fig-5]: Gel image showing positive band (121bp) for *Bordetella pertussis* IS481. 100 bp ladder (L); Lane 5: Positive sample; Lane 1,2,3,4,6: Negative sample; Lane 6: Negative control

Organisms	Target gene	Positive n (%)
Streptococcus pneumoniae	Lyt A	43 (21.50)
Haemophilus influenzae	16sRNA	2 (1.00)
Staphylococcus aureus	16sRNA	94 (47.00)
Klebsiella pneumoniae	16s-23S ITS	15 (7.50)
Bordetella pertussis	IS481	1 (0.50)

[Table/Fig-6]: Oropharyngeal (OP) swab PCR assay findings.

PCR results	Frequency (n)	Percentage (%)
Bordetella pertussis	1	0.50%
Klebsiella pneumoniae	15	7.50%
Staphylococcus aureus	92	46.00%
Staphylococcus aureus+Bordetella pertussis	1	0.50%
Streptococcus pneumoniae	42	21.00%
Streptococcus pneumoniae+Staphylococcus aureus	1	0.50%
Haemophilus influenzae	2	1.00%
Negative	46	23.00%
Total	200	100.00%

[Table/Fig-7]: Frequency of target organism by PCR assay including multiple organisms.
PCR: Polymerase chain reaction

Method of detection	Negative n (%)	Positive n (%)	p-value (χ^2)
Culture	79 (39.5)	121 (60.50)	<0.001* (11.08)
PCR	48 (24.0)	152 (76.0)	
Total	127	273	

[Table/Fig-8]: Comparison between oropharyngeal (OP) swab culture and PCR assay (N=200).
PCR: Polymerase chain reaction; *p<0.05 considered to be statistically significant; χ^2: Chi-square test

Study groups		Culture result				PCR result			
		Negative	Positive	Total	p-value	Negative	Positive	Total	p-value
Age group (months)	0-12	58 (39.46%)	89 (60.54%)	147 (73.50%)	0.93	32 (21.77%)	115 (78.23%)	147 (73.50%)	0.218
	>12	21 (39.62%)	32 (60.38%)	53 (26.50%)		16 (30.19%)	37 (69.81%)	53 (26.50%)	
Sex	Female	28 (36.84%)	48 (63.16%)	76 (38.00%)	0.55	14 (18.42%)	62 (81.58%)	76 (38.00%)	0.148
	Male	51 (41.13%)	73 (58.87%)	124 (62.00%)		34 (27.42%)	90 (72.58%)	124 (62.00%)	
Habitat	Rural	61 (38.12%)	99 (61.88%)	160 (80.00%)	0.43	38 (23.75%)	122 (76.25%)	160 (80.00%)	0.868
	Urban	18 (45.00%)	22 (55.00%)	40 (20.00%)		10 (25.00%)	30 (75.00%)	40 (20.00%)	
Socio-economic status	Lower middle class and above	42 (42.42%)	57 (57.58%)	99 (49.50%)	0.5	25 (25.25%)	74 (74.75%)	99 (49.50%)	0.907
	Upper lower class	24 (40.00%)	36 (60.00%)	60 (30.00%)		14 (23.33%)	46 (76.67%)	60 (30.00%)	
	Poor	13 (31.71%)	28 (68.29%)	41 (20.50%)		9 (21.95%)	32 (78.05%)	41 (20.50%)	

[Table/Fig-9]: Culture and PCR result in different demographic groups.
PCR: Polymerase chain reaction; p<0.05 considered to be statistically significant

DISCUSSION

Identification of the aetiological agent of CAP is important for proper treatment and thereby reducing mortality among the children under five years of age. Bacterial aetiology of CAP are routinely investigated by blood culture, sputum culture, serology for atypical bacteria (*Mycoplasma* species and *Chlamydophila* species), and pneumococcal antigen detection/PCR, as well as culture of pleural fluid/BAL/Lung aspirate etc., if samples are available. As it is challenging for microbiologist to identify the aetiological agent of CAP in children due to difficulty in obtaining uncontaminated OP swabs or lung tissue [15]; both culture method and PCR assay were used for identifying bacterial aetiology in the present study. Male:female ratio 1.6:1 (62% vs 38%) in the present study was concordant with the findings reported earlier by Ramachandran P et al., (58% boys and 42% girls) [16]. *Streptococcus pneumoniae* was the most common isolate recovered in this study which was in concordance with many previous studies [17,18]. Some earlier studies reported *S. pneumoniae and H. influenzae* to be the most common bacterial aetiological agents [19,20]. In a previous study, from north-east Indian region, Das A et al., reported *S. pneumoniae* and *H. Influenzae* to be the leading bacterial pathogens in under five children with CAP. They also reported notable finding of atypical bacterial pathogens *M. pneumoniae and C. pneumoniae* as causes of CAP [21]. Earlier Johnson AW et al., and Madhusudhan K et al., reported *S. aureus* and *K. pneumoniae* as commonest cause of pneumonia in a study in 2008 [22,23]. The only atypical bacterial pathogen found in the present study was *Bordetella pertussis* which was possible to detect only by PCR assay. Other atypical bacterial agents could not be detected by both the methods. Usually atypical bacteria are difficult to isolate by culture methods [24].

Though, *S. pneumoniae* is the most frequent cause of bacterial pneumonia in young children but is infrequently found in blood cultures [2]. The role of blood culture in diagnosis of CAP was studied by Tam PY et al., in their meta-analysis and reported with a positivity rate of 9.9% in hospitalised children with severe CAP [25]. The present study also found the isolation rate of blood culture as low as 13.5% which is concordant with above study [25]. In a recent study, Rodrigues CM and Groves H mentioned concomitant use of antimicrobials and localised infection of lung parenchyma to be the probable reasons for lower positivity rate of blood cultures in CAP [26]. Andrews AL et al., in 2015 also reported culturing of 118 blood samples to identify a single bacteremia [17]. Though, the Infectious Disease Society of America (IDSA)/ Paediatric Infectious Disease Society (PIDS) paediatric CAP guideline, 2011 recommended processing of blood cultures in children with moderate to severe CAP, but studies revealed low rate of bacteremia of 2.5-7% [27-29]. In 2015, Mathew JL et al., reported positivity rate of Nasopharyngeal Aspirate (NPA) and blood cultures in children as 13.7% and 2.1%, respectively. They isolated mostly *Streptococcus pneumoniae* (79.1%) in NPA while *S. aureus* (30.6%) in blood and reported *S. pneumoniae* as the predominant aetiological agent in NPA by culture as well as PCR; detection rate was higher in PCR than the culture [30]. Couple of earlier studies commented that aetiology of pneumonia can be identified only in 65-86% patients when multiple diagnostic tools including culture, serology and PCR are applied [2,31].

In the present study, PCR assay was found to be more useful in diagnosing the pathogen for bacterial pneumonia including those difficult to grow in conventional culture and was found to be statistically significant (p<0.001). Das A et al., in paediatric pneumonia and Stralin K et al., in adult pneumonia compared the performance of culture methods and PCR in detection of typical bacterial pathogen of pneumonia [21,32]. Authors did not find any significant statistical correlation between demographic factors and the diagnostic methods applied in this study; breast feeding and immunisation status could not be evaluated due to lack of evidence in most of the cases.

Limitation(s)

The fastidious and atypical bacterial pathogen could not be grown in culture though were detected in the PCR of OP swab. Serology for atypical bacteria could not be performed due to constraint of resources. Antibacterial susceptibility pattern of the culture isolates were not analysed which could have been useful in interpreting pattern of antimicrobial resistance, thus this study left the scope for future research. Also, authors could not correlate bacterial pathogen with clinical outcome of the CAP cases (recovery/death).

CONCLUSION(S)

Streptococcus pneumoniae and *Staphylococcus aureus* were the most common bacterial agents associated with CAP in children under five years. Also, application of PCR with bacterial specific primers in routine bacteriology laboratory can rapidly detect the fastidious pathogens such as *Haemophilus influenzae*, and atypical bacterial agents.

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