Original Article

Bacterial and viral etiology in hospitalized community acquired pneumonia with molecular methods and clinical evaluation

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Abstract

Introduction: Polymerase chain reaction (PCR) method has improved the diagnosis rates for patients with community-acquired pneumonia (CAP). We aimed to evaluate the bacterial and viral etiology of hospitalized CAP cases and compare clinical and laboratory findings of patients with pure bacterial and bacterial and viral (mixed) infections.

Methodology: A total of 55 patients hospitalized with CAP were enrolled into the prospective study between February 2010 and December 2010. Clinical and laboratory follow-up were performed on days 0, 7 and 14. Deep tracheal aspiration samples were examined for bacterial and viral pathogens by multiplex PCR, and standard bacteriological culture method.

Results: The etiological identification rate in 50 patients for bacteria, viruses and mixed virus–bacteria combination by PCR were 62%, 4%, 32%, respectively and 60% in 55 patients by bacterial culture method. *Streptococcus pneumoniae* concomitant with *Haemophilus influenzae* (36%) and rhinovirus (16%) was very common, whereas atypical pathogens (only *Mycoplasma pneumoniae*) were rare (6%). Rhinovirus was the most common viral agent (20%). Recently identified viruses, human coronavirus HKU1 and human bocavirus were not detected except for human metapneumovirus (one case). There was no significant difference in terms of mean age, immune status, leukocyte count, C-reactive protein (CRP) values, hospitalization duration and CURB-65 score between bacterial and mixed viral-bacterial detections. Advanced age ($p < 0.01$) and higher CURB-65 score ($p = 0.01$) were found to be associated with increased mortality.

Conclusion: Concomitance of bacterial and viral agents is frequent and resemble with bacterial infections alone. Further studies are needed for the clinical significance of mixed detections.

Key words: Adult; community acquired pneumonia; etiology; mixed infection; polymerase chain reaction; respiratory viruses

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Introduction

Community-acquired pneumonia (CAP) is responsible for a significant part of visits to physicians, treatment costs and deaths. Therefore, rapid diagnostic methods and pathogen-directed therapy are important. However, the etiology of CAP can be established in only 30-50% of cases using conventional methods [1] and unidentified etiology causes inappropriate antibiotic usage, antibiotic resistance, unintended adverse reactions and increased cost.

Sputum culture is often used for identification of probable/presumed etiology in CAP and it is recommended that the quality of respiratory samples should be determined by microscopy [2]. The diagnostic yield is influenced by antibiotic therapy, specimen collection, transport, rapid processing and correct use of cytological criteria [3]. Regarding viral and atypical pathogens, conventional culture methods require longer test times and a facility able to perform these tests. Nucleic acid tests with high sensitivity and specificity are advantageous for detection of fastidious or difficult to culture organisms [4]. In the last decade, both viral and mixed infections have been reported thanks to the increasing usage of molecular tests [5,6]. Multiplex PCR can detect several different agents in the same tube and offer rapid diagnosis. In addition it is not affected by antibiotic usage [4,7]. However, the

clinical impact of mixed infections has not been fully evaluated yet. Likewise, the role of viral infections in CAP are not well known in adults despite recent epidemics of severe viral pneumonia such as SARS, avian influenza, 2009 H1N1 influenza have focused attention on viruses [6].

The primary aim of this study was to evaluate the bacterial and viral etiology of CAP cases requiring hospitalization using multiplex PCR; whereas secondary objectives were to compare clinical and laboratory findings of patients with pure bacterial and mixed infections, and to compare standard bacterial culture and PCR methods.

Methodology

This prospective cohort study was approved by the Clinical Research Ethics Committee of Ege University Faculty of Medicine (10-4.1/9) and conducted in accordance with the amended Declaration of Helsinki. Written informed consent was obtained from all patients.

Inclusion criteria

Eighteen years of age or older**,** respiratory sample with leukocytes > 25 and squamous epithelial cells < 10 per low-power field with presence of previously reported criteria [8]: symptoms and signs of acute lower respiratory tract infection plus new infiltrates seen on a chest radiograph, with the absence of an alternative diagnosis.

Exclusion criteria

Below 18 years of age, healthcare-associated pneumonia, chronic obstructive pulmonary disease exacerbations without pneumonia, hospitalization and usage of antibiotics in the previous 48 hours.

Patient population

A total of 55 patients hospitalized with CAP admitted to Infectious Diseases or Chest Diseases or Emergency Medicine Departments at Ege University Faculty of Medicine between February 2010 and December 2010 were included in the study.

Case Report Form

Demographic information, clinical and laboratory data [leukocyte (normal range: $4–10\times10^3/\mu L$) and CRP (normal level: < 0.5 mg/dL) values] were collected on days 0, 7 and 14 after admission.

Respiratory sample collection and implantation in standard culture media

Respiratory samples were taken from each patient by protected deep tracheal aspiration and after Gram staining they were plated quantitatively on 5% sheep blood, chocolate and eosin methylene blue agars to investigate pathogen bacteria (*e.g. Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus* and Gram negative bacilli) within one hour after collection. Implanted plates were incubated at 37°C for 24-48 hours (cutoff $\geq 10^5$ CFU/mL). Conventional and automatic biochemical methods (VITEK 2, bioMérieux, Marcy l'Etoile, France) were used to identify isolated pathogens. The remaining part of the samples (with a volume of at least 3 mL) was stored at -80°C until PCR was performed.

Examination of samples with multiplex polymerase chain reaction method

Multiplex PCR method using dual priming oligonucleotide system for both bacteria (Seeplex PneumoBacter ACE Detection, Seegene Inc., Seoul, South Korea) and virus (Seeplex RV15 ACE Detection, Seegene Inc., Seoul, Korea) were used according to manufacturer's instructions. After nucleic acid extraction (Viral DNA/RNA extraction kit, Intron, Seoul, South Korea), cDNA was synthesized by reverse transcription (RevertAid first strand cDNA synthesis kit, Fermentas, Burlington, Canada) and used for virus detection as previously described [9]. PCR was performed in a total volume of 20 µL containing 3 µL of isolated nucleic acid solution, 4 µL 5× PneumoBacter primer or 4 µL of each 5× RV15 multiplex primer sets (A or B or C), 3 µL 8-methoxypsoralen contamination control reagent, and 10 µL 2× Multiplex Master Mix, as per the manufacturer's protocol. The amplification protocol was as follows: initial denaturation at 94℃ for 15 minutes, 40 cycles of denaturation at 94℃ for 30 seconds, annealing at 60℃ for 90 seconds, extension at 72℃ for 90 seconds, and a final extension step at 72°C for 10 minutes. Completed reactions were analysed using the Tape Station platform (Lab901, Edinburgh, UK). RV15 ACE PC, a mixture of 15 viral pathogens and internal control clones and PB ACE PC, a mixture of six bacterial pathogens and internal control clones were used as positive controls. The negative control contained sterilized water.

Pathogens in the virus kit

Set A: Human adenovirus A/B/C/D/E, Human coronavirus 229E/NL63, Human parainfluenza virus type 1/2/3, Set B: Human coronavirus OC43/HKU1, Human rhinovirus A/B/C, Human respiratory syncytial virus A/B, Influenza virus A, Set C: Human bocavirus 1/2/3/4, Influenza virus B, Human metapneumovirus, Human enterovirus, Human parainfluenza virus type 4

Pathogens in the bacteria kit

Mycoplasma pneumoniae, Legionella pneumoniae, S. pneumoniae, H. influenzae, Bordetella pertussis, Chylamydia pneumoniae.

Statistical methods

Data were analyzed using SPSS software, version 13.0 for Windows (SPSS). Chi square test was used for categorical variable and Student's *t* test was used for parametric measurements in the statistical analysis. A p value of < 0.05 was considered statistically significant.

Results

A total of 55 patients were included in the study. Five patients whose bacterial culture yielded *M. catarrhalis* and methicillin sensitive *S. aureus* were excluded for PCR evaluation since these pathogens did not exist on the PCR kit.

Patients' information

The mean age of 50 patients (19 female and 31 male) was 57.4 ± 18.9 (range 18–90) years. A total of 22 patients (44%) were immunocompromised. Thirty-six (72%) patients had at least one comorbid disease and 44 (88%) patients had at least one risk factor (Table 1). Mean CURB-65 [10] (Confusion, Urea nitrogen, Respiratory rate, Blood pressure, age ≥ 65 years) score of patients was 1.94 ±1.68 (range: 0-5). The average duration of hospitalization was 17 ± 15.9 (range 1-85) days. Mortality rate was 24% (12/50). Advanced age ($p < 0.01$) and higher CURB-65 score ($p = 0.01$) were associated with increased mortality. At admission, 72% of cases had leukocytosis (mean: $14.3\times10^3/\mu L$, standard deviation: 6.1) and 88% had high CRP levels (mean: 13.4 mg/dL, standard deviation: 13.5).

Microbiological etiology of CAP

Respiratory microorganisms were detected in 49 (98%) out of 50 patients by multiplex PCR method and the etiological identification rate was 62%, 4%, 32% for bacteria, viruses and mixed viral–bacterial combination, respectively. An etiologic agent was detected in 33 (60%) out of 55 patients by bacterial culture method. The bacterial culture results which yielded *S. pneumonia* and *H. influenza* were concordant with PCR results. *M. catarrhalis* (4 cases), methicillin sensitive *S. aureus* (1 case) and *P. aeruginosa* (1 case) were rare. Atypical organisms were identified in only 3 out of 50 (6%) patients (three *M. pneumoniae*, no *C. pneumoniae* or *L. pneumophila*) using PCR. Microbiological findings from bacterial culture and PCR method are presented in Table 2.

Viral pathogens were detected in 18 of 50 patients (36%) by PCR, and included rhinovirus (10 cases [20%]), respiratory syncytial virus A (4 cases [8%]), influenza A virus (2 cases [4%]), parainfluenza virus type 4 (2 cases [4%]), respiratory syncytial virus B (1 case [2%]), coronavirus 229E/NL63 (1 case [2%]), influenza B virus (1 case [2%]), human metapneuomovirus (1 case [2%]) and parainfluenza virus type 2 (1 case [2%]). Five patients had two viral agents (Table 3). Nine out of 10 patients with rhinovirus had a concomitant bacterial detection, and the most common combination was rhinovirus-*S. pneumoniae* (8 cases). Adenovirus, parainfluenza virus type 1 and 3, coronavirus OC43/HKU1, bocavirus and enteroviruses were not detected in any of the study patients.

There was no statistically significant difference in terms of age, immunocompromised status, duration of hospitalization, laboratory parameters, and CURB-65 score between mixed and isolated bacterial infections (Table 4). It was demonstrated that advanced age ($p < 0.01$) and high CURB-65 score ($p = 0.01$) had prognostic significance on mortality (Table 5). Bacterial and viral pathogens detected by conventional method or PCR according to CURB-65 score classes are shown in Table 6.

Table 1. Comorbid diseases and risk factors of patients with community-acquired pneumonia

	No. (%) of patients (n = 50)
Comorbid diseases[*]	
Cerebrovascular disease	11 (22)
Chronic obstructive pulmonary disease	9 (18)
Malignancy	8 (16)
Bronchiectasis	7 (14)
Diabetes mellitus	7 (14)
Congestive heart failure	7 (14)
Chronic renal failure	1 (2)
Liver disease	1 (2)
Risk factors[**]	
≥ 65 years	21 (42)
Smoking	20 (40)
Aspiration	12 (24)
İmmunosuppressive therapy	10 (20)
Corticosteroid therapy	8 (16)
Malnutrition	6 (12)
Pneumonia history during the previous year	5 (10)
Live in nursing home	4 (8)
Alcoholism	1 (2)
Splenectomy	1 (2)

[*]There were seven patients with two comorbid diseases and four patients with three comorbid diseases.
[**]There were 13 patients with two risk factors, eight patients with three risk factors and five patients with four risk factors.

Table 2. Bacterial and viral pathogens detected by conventional method and multiplex polymerase chain reaction method

	Conventional method (n = 55) No. (%) of patients	**PCR (n = 50) No. (%) of patients**
Bacterial pathogens		
Single bacterial pathogen		
S. pneumoniae	11 (20)	12 (24)
H. influenzae	12 (22)	6 (12)
M. catarrhalis	4 (7)	-
Methicillin sensitive *S. aureus*	1 (2)	-
M. pneumoniae	-	1 (2)
Multiple bacterial pathogens		
S. pneumoniae + H. influenzae	4 (7)	11 (22)
S. pneumoniae + H. influenzae + P. aeruginosa	1 (2)	*
S. pneumoniae + M. pneumoniae	**	1 (2)
Total	33 (60)	31 (62)
Viral pathogens		
Respiratory syncytial virus A	-	1 (2)
Rhinovirus	-	1 (2)
Total	-	2 (4)
Mixed bacterial-viral pathogens	***	16 (32)

* Conventional method result for *S. pneumoniae+H. influenzae+P. aeruginosa* was included in the calculation of *S. pneumoniae +H. influenzae* group with multiplex PCR.
** PCR result for *S. pneumoniae+M. pneumoniae* was included in the calculation of *S. pneumoniae* group with conventional method.
*** Mixed infections were calculated only by PCR results, because viral pathogens were identified using molecular method.
PCR: Polymerase chain reaction

Table 3. Mixed bacterial and viral pathogens detected by multiplex polymerase chain reaction method

	PCR (n = 50) No. (%) of patients
S. pneumoniae plus	
Rhinovirus	2 (4)
Influenza A virus	2 (4)
Parainfluenza virus type 4	1 (2)
Respiratory syncytial virus A	1 (2)
Parainfluenza virus type 4 + Rhinovirus	1 (2)
Respiratory syncytial virus A + *M. pneumoniae*	1 (2)
H. influenzae plus	
Influenza B virus + Rhinovirus	1 (2)
S. pneumoniae + *H. influenzae* plus	
Rhinovirus	2 (4)
Human metapneuomovirus	1 (2)
Coronavirus 229E/NL63	1 (2)
Respiratory syncytial virus A + Rhinovirus	1 (2)
Respiratory syncytial virus B + Rhinovirus	1 (2)
Parainfluenza virus type 2 + Rhinovirus	1 (2)
Total	16 (32)

Table 4. Comparative data between mixed bacterial-viral detections and pure bacterial detections

	Bacterial detections (n = 32)	Mixed detections (n = 16)	P value
Mean age (±SD), years	58.1 ± 18.7	54.1 ± 19.8	.5
İmmunocompromised status (n,%)	12 (24)	9 (18)	.46
Leukocyte count ($\times 10^3/\mu L$) on day 0 (±SD)	13.5 ± 6.4	16 ± 5.7	.19
Serum C-reactive protein level (mg/dL) on day 0 (±SD)	12.4 ± 13.2	15.4 ± 15.1	.52
CURB-65 score (±SD)	2 ± 1.6	1.56 ± 1.5	.37
Mean length of hospital stay (±SD), days	17.4 ± 14.9	11.6 ± 5.3	.053

SD: standard deviation; CURB 65: Confusion, Urea nitrogen, Respiratory rate, Blood pressure, age ≥ 65 years

Table 5. Prognostic factors on mortality

	Mortality (+) (n = 12)	Mortality (-) (n = 38)	P value
Mean age (±SD), years	73.6 ± 8.5	52.26 ± 18.4	<.01
CURB-65 score (±SD)	3.3 ± 1.4	1.5 ± 1.5	.01
Leukocyte count ($\times 10^3/\mu L$) on day 0 (±SD)	16.3 ± 5	13.7 ± 6.4	.20
Serum C-reactive protein level (mg/dL) on day 0 (±SD)	13.3 ± 9.6	13.4 ± 14.5	.98
Mean length of hospital stay (±SD), days	26.3 ± 27.4	14 ± 8.6	.15

SD: standard deviation; CURB 65: Confusion, Urea nitrogen, Respiratory rate, Blood pressure, age ≥ 65 years

Table 6. Bacterial and viral pathogens detected by conventional method or multiplex polymerase chain reaction method according to CURB-65 score

CURB-65 Score	Bacterial pathogens	Viral pathogens
1	*S. pneumoniae, H. influenzae*	Rhinovirus, Parainfluenza virus type 2 and 4, Influenza A virus, Respiratory syncytial virus A
2	*S. pneumoniae, H. influenzae, M. catarrhalis,* Methicillin sensitive *S. aureus*	-
3	*S. pneumoniae, H. influenzae, M. catarrhalis, M. pneumoniae*	Rhinovirus, Respiratory syncytial virus A
4	*S. pneumoniae, H. influenzae*	Rhinovirus, Parainfluenza virus type 4, Influenza B virus
5	*S. pneumoniae, H. influenzae*, *P. aeruginosa*	Influenza A virus

CURB 65: Confusion, Urea nitrogen, Respiratory rate, Blood pressure, age ≥65 years

Discussion

It is remarkable that 98% of patients were diagnosed with an etiological agent using PCR in our study. This is probably due to i) molecular tests which provided doubled increase in identification rate [6,11] ii) respiratory samples obtained by protected deep tracheal aspiration. This high detection rate through PCR is similar to the study in which respiratory microorganisms were detected in 97% of the children with CAP by Honkinen *et al.* [12]. Strålin *et al.* suggested that bronchoalveolar lavage multiplex PCR for *S. pneumonia*, *M. pneumoniae* and *C. pneumoniae* appears to be a useful etiological tool in lower respiratory tract infection patients and this method may be a valuable supplement to bronchoalveolar lavage culture [11]. Another probable reason for high etiologic agent recovery may be the severity of disease and the poor immune response in the elder patients requiring hospitalization. Templeton *et al.* reported increased identification rate with PCR and bacterial culture up to 87% in elderly patients and up to > 90% in patients with severe pneumonia [13]. Hence, advanced age, deteriorated general condition due to severity of pneumonia, immunosuppression or underlying diseases of our patients might have increased the etiologic agent isolation rate. In the presented cohort *S. pneumoniae* was the most commonly detected microorganism. This finding may be attributed to the use of invasive methods such as transtracheal aspiration and to the fact that many culture-negative cases of CAP are caused by pneumococci [5]. However, it should be considered that oropharyngeal contamination may have a role in high positive results. Contamination risk has been reported even in bronchoscopic materials [14]. The main issue with non-quantitative PCR is the difficulty in distinguishing colonization from infection due to frequent colonizers such as *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* [15,16]. Murdoch *et al.* suggested that this method may not be a reliable tool for diagnosing pneumococcal pneumonia on the grounds that 58% of control throat swabs were PCR positive [16]. This obstacle could potentially be overcome by the use of a quantitative PCR assay [17]. Rello *et al.* reported that determination of bacterial load may increase the diagnosis of pneumococcal pneumonia and may be useful for severity assessment [18].

In contrast to high identification rates, atypical organisms were rare and compliant with a recent study by Mermond *et al.* [19]. In another study from the same region of our country, atypical pathogens (most commonly *M. pneumonia,* 7%) were detected in 11.7% of 128 CAP patients [20]. The most likely reason for relatively low atypical pneumonia rate is progression of it in a milder manner as well as low requirement of hospitalization. It is also possible that there were no outbreaks during the period when samples were collected, or infection with atypical pathogens is negligible as mentioned by Creer *et al*. [21]. Due to complexity and poor reliability of the existing microbial culture and serological methods for the detection of these pathogens, specific investigation for them is rarely initiated. This probably results in underdetection and over empirical treatment regardless of a microbiological diagnosis [22]. Albeit with limited number of patients, our study contributes to the epidemiology of these pathogens in the hospitalized CAP patients.

There has traditionally been a limited focus on CAP viral causes until recently, based on the idea that they play only a minor role in adults [7]. The majority of studies have been performed with low sensitivity tests such as serology or fluorescent antibody tests and on a narrow spectrum of pathogens [13,23]. However, in the last decade, viral infections have been reported with increasing frequency by the use of molecular tests [6]. Rhinovirus is a well recognized cause of common cold. Nevertheless, its frequency may increase up to 33% especially in elderly and immunocompromised CAP patients [6,7,21]. The results of this study are in agreement with previous reports which describe significance of rhinovirus in severe lower respiratory disease [6,24]. The role of bocavirus as a true pathogen remains controversial and most studies have focused on young children and infants, yet at much lower positive rates older people are also susceptible to infection [25]. Likewise, in some studies there has been no correlation between coronavirus NL63/HKU1 and the disease whereas in other coronavirus OC43 has been detected as a cause for pneumonia at a rate of 2% [26-28]. Human metapneumovirus (hMPV) is a recently reported agent in studies using nucleic acid tests. Although Johnstone *et al.* [29] reported 3.6% of positivity in 198 CAP patients, Jennings *et al.* [6] did not report any positive cases in 304 patients. In our study, hMPV and coronavirus 229E/NL63 were detected in one patient each. Development of several multiplex assays has enabled simultaneous detection of up to 15 different viruses and the use of these tests is becoming standard for identification of respiratory viruses. Lower-respiratory specimens have obvious advantages for establishing the cause of pneumonia because they come from the site of infection [30]. The

fact that persistence of viral nucleic acids is relatively short-lasting in the respiratory epithelia [5] decreases the probability of colonization and suggests that viral detections in our study have clinical relevance. Hence, our data suggest that multiplex PCR greatly increases our ability to characterise the epidemiology of respiratory virus infections in adults.

Another significant finding of the present study was the high frequency of mixed viral–bacterial detection and it may refer to the fact that viral infections may induce bacterial infection. Furthermore, four to six pathogens are reported together in patients using molecular tests applied in other studies [12,21]. In our study rhinovirus and *S. pneumoniae* codetection was common in mixed infections as in the study of Kurutepe *et al;* which detected *S. pneumoniae* in mixed infections as copathogen in 11 out of 14 cases [20]. The underlying reason could be increased number of chronic obstructive pulmonary disease patients and smokers at advanced age, or detection of *S. pneumoniae* colonization. Although this condition complicates investigation of the role of each microorganism in the pathogenesis and clinical features, we speculated that mixed infection frequency may be higher in hospitalized cases in conjunction with the severity of disease. Several reports indicate that the incidence of mixed infections may be significant among patients admitted to hospital with CAP [5,8]. These patients are more likely to have underlying medical conditions and they may have a more severe course compared with monomicrobial pneumonia cases [31].

There was no statistical significant difference in terms of age, immunocompromised status, duration of hospitalization, laboratory parameters, and CURB-65 score between viral–bacterial co-detections and only-bacterial detections. In the study of Johnstone *et al.;* mortality, duration of hospitalization and acceptance rates to intensive care units were similar for both groups [29]. However, in other studies, concomitance of rhinovirus and pneumococci were found to be associated with severe pneumonia [6,13] and it was reported that patients infected with a virus and a bacterial pathogen more often develop severe CAP with a longer hospitalization [32]. In our study, further evaluation could not be performed due to the low number of patients. Our results do not support the use of CRP level and leukocyte count as a rapid test to distinguish mixed infections in patients with CAP requiring hospitalization.

The most important limitation of our study is the relatively small number of patients for external validity of the study. It is worth to state that exclusion of COPD exacerbations without pneumonia may be an important reason for the low patient number. There was no control group to evaluate the significance of detection of *S. pneumoniae* and *H. influenzae* using the present PCR kit. We had no chance to evaluate colonization of the upper respiratory tract or infection, yet we are informed of atypical and viral pathogens. Another important limitation is that only hospitalized patients were included into the study. Hence, there can be differences in the distribution of pathogens between inpatients and outpatients. Subgroup analysis could not be performed due to the low number of patients. Moreover, the samples were gathered and a batch study was performed, so they did not have active contributions in directing patients' treatment, but provided implications retrospectively.

S. pneumoniae and *H. influenzae* are well recognized as causes of pneumonia and they are the most commonly detected agents. However, conventional diagnostic methods often fail to identify atypical and viral respiratory agents, and the frequency of these pathogens in adults is uncertain. In our study, albeit with a small number of patients, the PCR test defined the epidemiology of these pathogens in CAP.

Conclusions

In the present study pathogens were detected in 98% of patients by using a molecular method. Mixed viral–bacterial detections were more common than expected in CAP requiring hospitalization and resembled individual bacterial infections in clinical and laboratory examinations. Viruses may play an important role in CAP etiology. These data also suggest that initial empirical antibiotic treatment in patients with hospitalized CAP does not need to cover atypical organisms.

PCR method provides simply rapid results especially for viral and atypical agents. However, further investigations are needed to evaluate whether the detection of bacteria is the causative agent and if treating PCR positive patients is beneficial or not. A high concentration of bacteria detected by quantitative PCR may represent clinically significant infection.

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