Parallel pathogens in the upper and lower respiratory tracts in children with a respiratory tract infection, as revealed by the Filmarray assay

Yongping Lin [a,1], Weiwen Liang [a,1], Danyun Miao [a], Dingqiang Chen [a], Shangzhi Wu [b], Zhiying Ye [b], Zhongmin Liu [a,*], Dehui Chen [b,*]

[a] Department of Clinical Laboratory, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou 510120, China
[b] Department of Pediatric, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou 510120, China

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ABSTRACT

Background: Respiratory tract infection (RTI) is a common disease among children of all ages that causes high hospitalization and mortality rates. Infection with more than one pathogen has been reported in RTI; however, the association of the pathogen spectrum in upper and lower respiratory tract infections remains unclear.

Methods: A prospective study was conducted during February to October 2016. Fifty-five nasopharyngeal swabs (NPS) and 30 bronchoalveolar lavage fluid (BALF) samples from 55 hospitalized children aged less than 14 years (mean age 40 months) and diagnosed with an RTI were collected. All samples were detected for 18 respiratory pathogens using the Filmarray assay, real-time PCR, or nested PCR methods. Detection results and clinical characteristics of all cases were analyzed using chi-square and t tests.

Results: Forty-one of 55 (74.5%) NPS obtained from children were positive for at least one pathogen by the Filmarray assay. Of these cases, 53.7% (22/41) were co-infected. The most commonly detected pathogen was rhinovirus (RV), followed by Mycoplasma pneumoniae (MP) and respiratory syncytial virus (RSV). Infection by both RV and MP was the most frequently observed pattern of co-infection. Similar results were observed using real-time PCR. The pathogens in the NPS from 76.6% of cases detected by Filmarray and 80.0% of cases by real-time PCR included all the pathogens detected in the BALF sample from the same individual. The Filmarray assay showed an 80% concordance rate with real-time PCR and had a turnaround time of less than 1.2 h. No significant differences were observed between the association of single-infection and co-infection with clinical characteristics, neither by Filmarray nor real-time PCR.

Conclusion: The spectrum of pathogens is mostly concordant in the upper and lower respiratory tract. Collecting NPS for detection can be a non-invasive and more convenient option compared with BALF. Although co-infection is common in children with an RTI, the clinical significance of co-infection remains unclear and warrants further analysis.

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Introduction

Respiratory tract infection (RTI) is a common disease in all ages of the population, especially in children. The severity of RTI varies from mild pharyngolaryngitis to pneumonia or even death. RTIs result in high pediatric hospitalization and morbidity rates. In 2013, up to 0.9 million children aged under 5 years died from pneumonia globally, which ranks as the second leading cause of childhood death.[1] Rhinovirus (RV), Mycoplasma pneumonia (MP), adenovirus (ADV), and parainfluenza virus (PIV) were reported to be detected commonly in RTIs.[2–5] Traditional detection methods, such as viral or bacterial culture, usually require 3–5 days and depend on the biological properties of the pathogens.[6] Immunoassays provide faster diagnosis for pathogens but suffer from poor specificity of the antigen–antibody reactions.[7] With the development of molecular diagnosis techniques, PCR-based methods with preferable sensitivity and efficiency can detect co-infection, which is common in children with an RTI.[8–10] Filmarray is a nested

PCR-based platform for pathogen detection. It can complete the detection of 18 respiratory pathogens automatically, with good sensitivity and specificity within 1.2 h[9,11,12]

Differences in the pathogen spectrum between upper and lower respiratory tract infections remain controversial. It has been demonstrated in several studies that RV, respiratory syncytial virus (RSV), ADV, and MP were detected frequently in both nasopharyngeal swabs (NPS) and bronchoalveolar lavage fluid (BALF) from patients with an RTI.[13–16] However, some pathogens, such as influenza A virus, were detected more frequently in NPS than in BALF.[17,18] An *ex-vivo* study by Nicholls et al. demonstrated that Influenza A virus (H5N1) could not only infect, but also replicate in tissue from the human upper respiratory and lower respiratory tract.[19] There may be an association between the pathogen spectrum in the upper respiratory tract and lower respiratory tract; however, the different detection rates for pathogen between NPS and BALF might be attributed to the tropism of pathogens in respiratory tract. Few studies have demonstrated the pathogen spectrum in the upper and lower respiratory tract of the same individual. In the present study, we used the Filmarray assay and real-time PCR methods for pathogen detection to investigate pathogens in children with an RTI. Furthermore, we detected pathogens in the NPS and BALF from the same individual, which could help to better understand pathogen distribution and association between the upper respiratory and lower respiratory tract.

Material and methods

Inclusion criteria and sample collection

A prospective study was conducted in hospitalized children with an RTI at the First Affiliated Hospital of Guangzhou Medical University. The research was approved by the ethical committee of the hospital. During February to October 2016, children aged less than 14 years, presenting influenza like symptoms (including fever and cough) and hospitalized with an RTI were enrolled in this study. The enrolled children comprised 33 males and 22 females, with a mean age of 40 months. NPS were collected from each patient and stored in transport medium, according to the manufacturer's instructions of the Filmarray (Biomerieux, Lyon, France). Thirty BALF samples were also collected from 30 of the 55 children undergoing fibrobronchoscopy. All samples were vortexed and centrifuged at 8000 rpm for 5 min to yield cell-free supernatant for immediate detection by the Filmarray. A portion of the supernatant were stored at −80 °C for detection by real-time PCR.

Pathogen detection by the Filmarray respiratory panel assay

The Filmarray assay was performed for respiratory tract pathogen detection following the manufacturer's instructions.[20] Eighteen pathogens were detected in each run of the Filmarray assay, including RV, MP, RSV, PIV1, PIV2, PIV3, PIV4, influenza A virus (IFA), influenza B virus (IFB), enterovirus (EV), adenovirus (ADV), coronavirus 229E (CoV 229E), CoV NL63, CoV HKU1, CoV OC43, human metapneumovirus (MPV), *Chlamydophila pneumoniae* (CP), and *Bordetella pertussis* (BP). The results were analyzed automatically by the Filmarray software. Only those results that were positive for two internal controls were considered valid for analysis.

Pathogen detection by real-time PCR

Total nucleic acid was extracted from 140 μL of each specimen following the manufacturer's protocol of the QIAamp Viral RNA Kit (Qiagen, Valencia, CA, USA). Reverse transcription was then per-

formed according to the protocol provided by the Prime Script RT Master Mix Kit (Takara Bio, Otsu, Japan). Specific primer pairs and probes were designed to target genes of the pathogens or modified according to the literature (Table 1).

Verification of inconsistent results by the nested PCR

Samples with inconsistent detection results by Filmarray and real-time PCR were verified by nested PCR according to a previous study.[28]

Statistics

Statistics analysis was performed using SPSS 17.0 software. The detection rates for pathogens in NPS were calculated. Correlation between the pathogen or co-infection rate and the clinical characteristics were analyzed using the chi-square test and a *t* test. A *P* value <0.05 was considered to be statistically significant.

Results

Pathogens in the upper respiratory tract of children with an RTI

In the 55 NPS detected by the Filmarray, 41 (74.5%) were positive for at least one pathogen. Furthermore, co-infection was observed in 22 of 41 (53.7%) infected children. Data for the 55 children were categorized into three groups according to age (Table 2). Neither the pathogen infection rate nor the co-infection rate showed a significant difference among the three groups. However, children aged less than 2 years tended to be more susceptible to infection than older children.

RV and MP were the most common pathogens, found in 38.2% and 29.1% of NPS detected by the Filmarray, respectively. Moderate detection rates were observed for RSV (12.7%), ADV (12.7%), PIV3 (10.9%), and NL63 (9.1%). IFA, IFB, PIV1, PIV4, and BP were detected rarely, with detection rates lower than 5.6%. No case was positive for MPV, PIV2, CoV 229E, CoV OC43, CoV HKU, CP, or EV in this study. The detection rates for each pathogen were compared between children aged less than 2 years and those aged over 2 years (Fig. 1). MP was detected more frequently in children aged over 2 years (*P* < 0.01), while RSV was detected more frequently in children aged less than 2 years (*P* < 0.01). The most prevalent pathogen involved in co-infections was RV: 72.7% of co-infections involved RV. MP was observed in 50% of the co-infections. Among all pathogens detected in this study, co-infection by RV and MP was the most frequent co-infection pattern: 27.3% of co-infections comprised RV and MP (Table 3).

Real-time PCR detection of the 55 NPS samples showed similar results, with a pathogen detection rate of 74.5% (41/55). Seventeen of 41 (41.5%) were co-infections. The most commonly detected pathogens were RV and MP, which were found in 34.5% and 29.1% of NPS, respectively (Supplementary Tables 1 and 2 and Supplementary Fig. 1).

Pathogen distribution in the upper and lower respiratory tract

NPS and BALF collected at the same time from 30 children were detected by the Filmarray and real-time PCR. The pathogens in 30 NPS and BALF samples detected by the Filmarray are listed in Table 4. Pathogens in the NPS from 23 (76.6%) children included all the pathogens detected in the BALF samples from the same individual. In these 23 cases, 16 (69.6%) had exactly the same pathogens in both the NPS and BALF samples. Among all the RV-infected cases, RV was always detected in NPS as well as in BALF,

Table 1
Primers and Probes specific for target genes of pathogens and used in real-time PCR.

Pathogen	Target gene	Sequences of primers/probes (5′–>3′)	Citation
IFA	Matrix	F: GACCRATYCTGTCACCTCTGAC R: AGGGCRTTYTGGACAAAVCGTCTA P: TGCAGTCCTCGCTCACTGGGCACG	Ref. [21] modified
IFB	Matrix	F: ACACAGRGCTCATAGCAGAG R: ATGTTGCTTTGCAGYTCTTCTGC P: CGAGATCYTCAGTGCCYGGAGTGAG	This study
IFA-H1N1	HA	F: CTGGGAAATCCAGAGTGTGAATC R: CTGGGTAACACGTTCCATTGTCT P: CTCTCCACAGCAAGCTCATGGTCCTAC	This study
PIV1	HN	F: GTTGTCAATGTCTTAATTCGTATCAATAATT R: GTAGCCTMCCTTCGGCACCTAA P: TAGGCCAAAGATTGTTGTCGAGACTATTCCA	Ref. [22] modified
PIV2	HN	F: GCATTTCCAATCTTCAGGACTATGA R: ACCTCCTGGTATAGCAGTGACTGAAC P: CCATTTACCTAAGTGATGGAATCAATCGCAA	Ref. [22] modified
PIV3	HN	F: CCRGATGGRTGTATAACRGGAG R: GTTATGACTGGGTTRACTCTCG P: CYGATGCATATCCACTCAATCCCACAGG	This study
PIV4	HN	F: TGACACTCAACAAATYAAAGGTTCA R: ACTCCAGGRTCCATTATTTTCATTG P: TTGCMACAATTGAGGGCCTAATCAC	Ref. [15]
RV	polyprotein	F1: GTGTGAAGAGCCSCRTGTGCT F2: GGTGTGAAGAGYCTAKTGTGCT R: ACGGACACCCAAAGTAGTYGGT P: TCCGGCCCCTGAATGYGGCTAAYC	Ref. [23] modified
MP	P1	F: GGAATCCCAATGCACAAGAACA R: GCTTTGGTCAACACATCAACCTT P: CCTTGAAGGCTGGGTTTGCGCTA	Ref. [23]
RSV	P	F: GCAAATATGGAAACATACGTGAACA R: GCACCCATATTGTWAGTGATGCA P: CTTCACGARGGCTCCACATACACAGCWG	Ref. [23]
ADV	Hexon	F: GCCACGGTGGGGTTTCTAAACTT R: GCCCCAGTGGTCTTACATGCACATC P: TGCACCAGACCCGGGCTCAGGTACTCCGA	Ref. [24]
EV	polyprotein	F: CATGGTGYGAAGAGTCTAYTGAGC R: GGACACCCAAAGTAGTCGGTTC P: CGGCCCCTGAATGCGGCTAATC	Ref. [23]
MPV	NP	F: GAATCATCAGGCAATATTCCAC R: AGTGCATCACTTAGYACACGG P: CAGAGRCCTTCAGCACCAGACACACC	Ref. [23]
CP	MOMP	F: CAAGGGCTATAAAGGCGTTGCT R: ATGGTCGCAGACTTTGTTCCA P: TCCCCTTGCCAACAGACGCTGG	Ref. [23]
Cov NL63	1A	F: ACGTACTTCTATTATGAAGCATGATATTAA R: AGCAGATCTAATGTTATACTTAAAACTACG P: ATTGCCAAGGCTCCTAAACGTACAGGTGTT	Ref. [25]
Cov OC43	NP	F: CGATGAGGCTATTCCGACTAGGT R: CCTTCCTGAGCCTTCAATATAGTAACC P: TCCGCCTGGYACGGTACTCCCT	Ref. [25]
Cov 229E	NP	F: CAGTCAAATGGGCTGATGCA R: AAAGGGCTATAAAGAGAATAAGGTATTCT P: CCCTGACGACCACGTTGTGGTTCA	Ref. [25]
Cov HKU1	Lab	F: CCATTACAAGCCATAAGAGAACAAAC R: TATGTGTGGCGGTTGCTATTATGT P: TTGCATCACCACTGCTAGTACCACCAGG	Ref. [26]
BP	IS481	F: CAAGGCCGAACGCTTCAT R: GTTCTGGTAGGTGTGAGCGT P: CAGTCGGCCTTGCGTGAGTGGG	Ref. [27] modified

Table 2
Detection rate for pathogens in NPS from children with an RTI, as detected by the Filmarray.

	Aged less than 2 years ($n = 26$)	Aged 2 to 6 years ($n = 20$)	Aged 7 to 14 years ($n = 9$)	Total ($n = 55$)
Pathogen positive	22 (84.6%)	14 (70.0%)	5 (55.6%)	41 (64.5%)
Co-infected cases	11 (50.0%)	8 (57.1%)	3 (60.0%)	22 (53.7%)

which showed a concordance of 100% between the upper and lower respiratory tract samples. The pathogen detection rate in the NPS and BALF samples was 73.3% and 80.0% respectively. The co-infection rate detected in the NPS and BALF samples was 40.0% and 33.3% respectively.

Pathogens detected in the 30 NPS and BALF samples using real-time PCR are shown in Supplementary Table 3. Pathogens in the NPS from 24 of 30 (80.0%) cases included all the pathogens detected in the corresponding BALF sample. Twenty of 24 cases (83.3%) had the same pathogens in the NPS and in BALF samples.
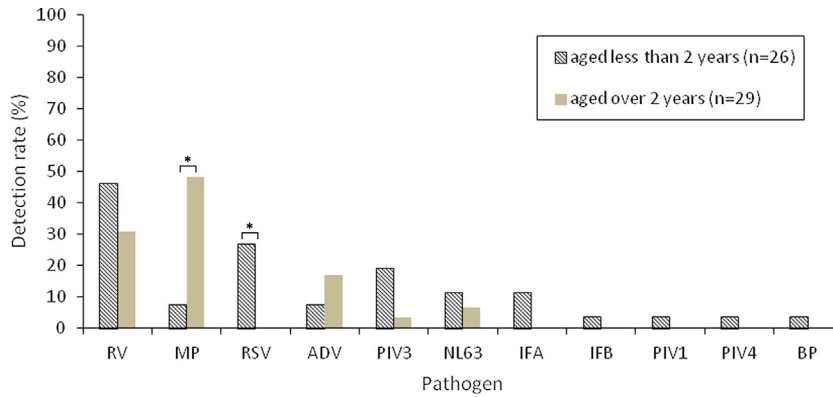
Fig. 1. Detection rates for 11 pathogens (RV, MP, RSV, ADV, PIV3, CoV NL63, IFA, IFB, PIV1, PIV4, and BP) in children aged less than 2 years and those aged over 2 years, as assessed by the Filmarray. Analysis was performed using a Chi-square test. Statistical significance is indicated by asterisks: $^{*}P < 0.01$.

Correlation between single-infection or co-infection with the clinical characteristics

The clinical characteristics of the 55 children that provided an NPS that was detected by Filmarray were analyzed. The major clinical characteristics including mean age, fever, cough, nasal obstruction, rhinorrhoea, dyspnea, shortness of breath, pleural effusion, radiography diagnosis as pneumonia, severe pneumonia, and complication were compared between single-pathogen infections (SI) and co-infections (CI) (Table 5). No significant difference was found between single pathogen infections and co-infections. Similar results were observed using real-time PCR (Supplementary Table 4).

Differences in the detection results between the Filmarray and real-time PCR

Fifty-five NPS and 30 BALF samples were detected by the Filmarray and real-time PCR. Twelve pathogens were identified by the Filmarray in this study, including RV, MP, RSV, ADV, PIV3, NL63, IFA (H1N1, H3N2), IFB, PIV1, PIV4, and BP, while 11 were detected by real-time PCR (PIV4 was negative). Of the 85 samples detected, 71 (83.5%) samples showed consistent results by the two assays. Of the 14 inconsistent samples, 12 samples were detected as having more pathogens by the Filmarray than by real-time PCR. All 14 samples were further verified by nested PCR method. Two samples were proven to be consistent after verification by nested PCR, while the other 12 samples remained inconsistent. The concordance rate was 85.9% after verification by nested PCR (Table 6).

Discussion

In this study, pathogens in 55 NPS and 30 BALF samples obtained from 55 hospitalized children with an RTI were detected. Using the Filmarray, 74.5% of the children were found to be infected with at least one pathogen, among which 53.7% of cases were co-infected. Co-infection has been reported to be common in children with an RTI. Previous studies showed that co-

Table 4
Pathogens in NPS and BALF of 30 children detected by Filmarray.

Patient	In NPS	In BALF	Patient	In NPS	In BALF
1$^{*\triangle}$	RV, PIV3	RV, PIV3	16$^{*\triangle}$	Negative	Negative
2$^{*\triangle}$	RV, NL63	RV, NL63	17*	MP, ADV	MP
3$^{*\triangle}$	RV, MP	RV, MP	18*	RV, ADV	RV
4$^{*\triangle}$	MP	MP	19*	RV, MP, PIV3	RV, MP
5$^{*\triangle}$	MP	MP	20*	NL63, MP	MP
6$^{*\triangle}$	RSV	RSV	21*	RV, PIV4	RV
7$^{*\triangle}$	RSV	RSV	22*	RV, NL63, PIV3	RV
8$^{*\triangle}$	RV	RV	23*	MP, ADV	MP
9$^{*\triangle}$	RV	RV	24	RV, RSV	RV, RSV, MP
10$^{*\triangle}$	RV, ADV	RV, ADV	25	MP	MP, NL63
11$^{*\triangle}$	Negative	Negative	26	RV	RV, ADV
12$^{*\triangle}$	Negative	Negative	27	RSV	RSV, BP
13$^{*\triangle}$	Negative	Negative	28	ADV	ADV, MP
14$^{*\triangle}$	Negative	Negative	29	Negative	MP
15$^{*\triangle}$	Negative	Negative	30	Negative	PIV3

The pathogens in the NPS and BALF from the same individual were compared. Cases with asterisk ∗ show that the pathogens in the NPS can include the pathogens in the BALF. Cases with triangles Δ show that the pathogens were completely consistent in NPS and BALF.

Table 3
Pathogens detected in multi-analyte-positive samples by the Filmarray.

Analyte 1	Analyte 2	Analyte 3	Analyte 4	No. of cases (%)
RV	MP			5 (22.7)
RV	MP	PIV3		1 (4.5)
RV	ADV			3 (13.6)
RV	RSV			1 (4.5)
RV	RSV	PIV3		1 (4.5)
RV	PIV3			1 (4.5)
RV	PIV3	NL63		1 (4.5)
RV	PIV3	PIV1	BP	1 (4.5)
RV	PIV4			1 (4.5)
RV	NL63			1 (4.5)
MP	ADV			2 (9.1)
MP	NL63			2 (9.1)
MP	NL63	IFA (H1N1)		1 (4.5)
RSV	PIV3			1 (4.5)

Table 5
Clinical characteristics of 55 children having NPS detected by the Filmarray.

Characteristics	No. (%) detected by Filmarray		P value
	SI ($n = 19$)	CI ($n = 22$)	
Mean age (month)	32.6 ± 35.8	35.8 ± 31.7	P > 0.05
Fever	18 (94.7)	22 (100.0)	P > 0.05
Cough	18 (94.7)	21 (95.5)	P > 0.05
Nasal obstruction	6 (31.6)	3 (13.6)	P > 0.05
Rhinorrhoea	7 (36.8)	3 (13.6)	P > 0.05
Dyspnea	10 (52.6)	8 (36.4)	P > 0.05
Shortness of breath	13 (68.4)	9 (40.9)	P > 0.05
Diagnosed as pneumonia	16 (84.2)	21 (95.5)	P > 0.05
Severe pneumonia	6 (31.6)	4 (18.2)	P > 0.05
Pleural effusion	2 (10.5)	3 (13.6)	P > 0.05
Complication	0 (0.0)	1 (4.5)	P > 0.05

SI: single-pathogen infections; CI: co-infections.

Table 6
Samples with inconsistent results detected by Filmarray, real-time PCR and verified by nested PCR.

No.	Sample type	By Filmarray	By real-time PCR	Verification by nested PCR
1	NPS	Negative	H3N2, IFB	H3N2, IFB
2	NPS	Negative	IFB	IFB
3	NPS	RV, RSV, PIV3	RV, RSV	RV, RSV
4	NPS	RSV, PIV3	PIV3	PIV3
5	NPS	RV, MP	MP	MP
6	NPS	RV	Negative	Negative
7	NPS	RV, ADV	RV	RV
8	NPS	RV, PIV4	RV	RV
9	NPS	ADV, MP	MP	MP
10	NPS	RV, ADV	RV	RV
11	NPS	RSV	Negative	RSV
12	BALF	RSV	Negative	RSV
13	BALF	RV, ADV	RV	RV
14	BALF	RV, RSV, MP	RV, RSV	RV, RSV

infection was found more frequently in children under 2 years old than in older age groups.[4,5,10,20,29,30] However, Peng et al. stated that children aged from 3 to 6 years might be more susceptible to co-infection.[31] This may be explained by the increased exposure to shared childcare groups. However, our study showed no significant increase in co-infection in children aged over 2 years. The correlation between co-infection and age in children with an RTI remains controversial and requires further study.

RV was the most prevalent pathogen overall and in the co-infection cases in this study. Other studies showed similar results, which highlighted the important role of RV in RTIs.[3,20,30] RSV and MP were also found commonly in children with an RTI. Our study showed that children younger than 2 years old were more susceptible to RSV, which agreed with other studies.[3,32,33] In addition, MP was detected more frequently in children aged over 2 years. Some studies also showed that MP was detected more commonly in pre-school-aged children and adolescents with community-acquired pneumonia.[34,35] Our findings may improve diagnosis and lead to better clinical care.

The Filmarray, which is approved by the Food and Drug Administration (FDA), is a platform that uses NPS for detection. In this study, the Filmarray has been used to demonstrate that the spectrum of pathogen was similar between upper and lower respiratory tract of patients with an RTI, which agreed with other studies.[13–18] Few studies have investigated the spectrum of pathogens in the respiratory tract from the same individual. In 24 out of 30 cases for which NPS and BALF samples were available in this study, the pathogens in the NPS included all the pathogens detected in the BALF sample from the same individual. Our findings suggested that although the tropism of several pathogens might contribute to the different detection rates in the upper and lower respiratory tract, the spectrum of pathogens is mostly concordant in the upper and lower respiratory tract. The pathogens detected in the NPS can predict well the pathogens in the lower respiratory tract. Thus, collecting NPS for pathogen detection could be a non-invasive and preferable option.

The Filmarray assay has been used to diagnose RTIs in previous studies.[20,36,37] It could detect co-infection efficiently, with good sensitivity, and showed more than 80% concordance with the real-time PCR results in this study. The overall turnaround time

of the Filmarray is shorter than routine PCR. Fast and comprehensive detection for respiratory pathogens helps to avoid the abuse of antibiotics and leads to better clinical care.

The clinical characteristics between single-infections and co-infections showed no significant differences based on the observational data in our study. A previous study also demonstrated that neither viral load nor viral co-infections were significantly associated with disease severity.[3,10,32] The study of co-infection and the clinical features in children with RTIs were limited by the number of participants in this study. Further study is required to better understand the relevance the data presented here.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.flm.2017.02.004.

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