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Research Article

Development of In-House Multiplex PCR Assays to Detect Respiratory Microorganisms and Implementation using Nasopharyngeal Swabs from Rural Senegal

Dame Ndiaye[1,2,3], Marielle Bedotto-Buffet[4], Georges Diatta[3], Hubert Bassene[3], Sébastien Cortaredona[1,2,3,5], Masse Sambou[3], Sophie Edouard[1,2], Oleg Mediannikov[6,7], Pierre-Edouard Fournier[1,2], Cheikh Sokhna[1,2,3,5], Florence Fenollar[1,2*]

[1]Aix Marseille Univ, AP-HM, SSA, RITMES, Marseille, France

[2]IHU-Méditerranée Infection, Marseille, France

[3]EMR MINES, IRD 279, Campus Commun UCAD-IRD Hann, Dakar, Senegal

[4]AP-HM, Marseille, France

[5]Aix Marseille Univ, IRD 279, SSA, MINES, Marseille, France

[6]Aix Marseille Univ, AP-HM, MEPHI, Marseille, France

[7]IRD, Marseille, France

[*]**Corresponding author:** Florence Fenollar, IHU-Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13005 Marseille, France

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Abstract

Acute respiratory infections are a common and serious public health problem worldwide, especially in resource-limited countries. Although highly multiplexed molecular diagnostic tests allow clinical microbiology laboratories to rapidly detect a wide range of respiratory microorganisms, these commercial tests are expensive. Our aim was to develop affordable in-house multiplex PCR assays and apply them to nasopharyngeal samples from Senegalese patients to assess local epidemiology. Based on available simplex PCR systems, we developed in-house duplexed and triplexed PCR assays targeting 23 respiratory pathogens. Their specificity and sensitivity were evaluated using 965 DNA/RNA extracts from nasopharyngeal swabs, by comparing the results obtained with the same systems in simplex PCR and with a commercial kit (FTD® Respiratory Pathogens 21) used for routine diagnosis in our French laboratory. In-house multiplexed assays were then applied to swabs from 500 febrile patients from the rural area of Niakhar. In-house multiplex tests had a specificity and sensitivity of 100%, with an estimated cost of which was three to 6.5 times lower than commercial kits and an analysis time of two hours. *Streptococcus pneumoniae* was the most frequently observed bacterium (n=181; 36.2%). For respiratory viruses, adenovirus was the most prevalent (n=26, 5.2%), followed by RSV (n=15, 3%), SARS-CoV-2 (n=11, 2.2%), rhinovirus (n=10, 2%) and influenza A virus (n=9, 1.8%). Co-infection was detected in 28.2% (n=141) of nasopharyngeal samples. In-house duplex and triplex PCR assays are efficient and affordable. Their use can be modular, targeting the most frequent pathogens according to local epidemiology in order to further limit costs.

Keywords: *Streptococcus pneumoniae*; Adenovirus; RSV; Respiratory microorganisms; Senegal; Multiplex qPCR assays

Introduction

Acute respiratory infections are a critical public health concern worldwide, particularly in countries with limited resources [1]. They are very common and ubiquitous, affecting adults and children alike [2]. The spectrum of microorganisms involved in acute respiratory infections is vast, including bacteria such as *Streptococcus pneumoniae* and viruses such as influenza viruses, respiratory syncytial viruses (RSV), rhinovirus, enteroviruses, metapneumovirus (hMPV), adenovirus (AdV), human bocavirus, parainfluenza (HPIV), MERS-CoV, coronaviruses (HCoV-E229, HCoV-OC43, HCoV-NL63, HCoV-HKU1), and SARS-CoV-2 since 2020 [3]. They are one of the main causes of consultations in health centres, morbidity, hospitalisation, and mortality [4], with impacts worldwide, as evidenced by the COVID-19 pandemic [5]. Infection with the influenza A virus also causes significant morbidity and mortality worldwide, despite vaccination and antiviral treatment [6]. Acute respiratory infections, particularly lower respiratory tract infections, are the leading cause of death among children under the age of five and are estimated to be responsible for between 1.9 million and 2.2 million childhood deaths globally every year. Forty-two percent of these acute respiratory infection-associated deaths occur in Africa [4]. In Senegal, they remain the leading cause of infectious mortality [7]. The diagnosis of infectious diseases currently relies on a range of laboratory methods, including culture, serological tests, nucleic acid amplification tests, antigen detection, and direct visualisation [8]. Isolating and identifying pathogens is a delicate task, and not within the reach capabilities of all healthcare facilities [9]. Moreover, a number of infectious diseases, many of them emerging and/or neglected, can only be reliably diagnosed by molecular biology [10]. Over the past fifteen years in Senegal, we have been studying the causes of non-malarial fevers using real-time PCR assays performed on blood extracts [11,12]. We first established a repertory of microorganisms, and then set up our first Point-Of-Care (POC) laboratory at the Dielmo-Ndiop dispensary, followed by another at the Niakhar dispensary. Several bacteria were identified by molecular biology, including Rickettsia species [13], *Borrelia crocidurae* [14], *Tropheryma whipplei* [11,15], and *Coxiella burnetii* [16]. With the onset of the COVID-19 pandemic, these POC laboratories began to contribute to the on-site molecular diagnosis and monitoring of SARS-CoV-2 in the rural areas of Niakhar, Dielmo, and Ndiop in Senegal [17].

Diagnostic methods have evolved significantly, with the advent of molecular biology techniques and, more particularly, multiplex molecular tests, which allow the simultaneous detection of a large number of infectious agents. These tests are, however, expensive and unsuited to low-income countries [2].

Our aim was to develop and evaluate in-house duplex and triplex PCR assays targeting a wide range of respiratory microorganisms, including bacteria and viruses, as well to complete the repertoire of respiratory microorganisms in the Niakhar area of rural Senegal.

Material and Methods

Origin of nasopharyngeal samples and ethical aspects

The specificity and sensitivity of the in-house duplexed and triplexed molecular systems were evaluated, firstly, on the basis of the "*tube bottoms*" of French samples, by comparing the results obtained with the same systems in simplex PCR and with a commercial kit used for routine diagnosis in our clinical microbiology laboratory. Then, the in-house duplexed and triplexed molecular systems were applied to Senegalese samples from a rural area of interest.

The French specimens corresponded to "*tube bottoms*" from nasopharyngeal swabs sampled as part of care and sent for the diagnosis of respiratory infections in our infectious agent diagnostic laboratory at the Marseille University Hospitals (AP-HM, Marseille). In accordance with French regulations, during their hospitalisation or consultation, patients are informed by the establishment of the possibility of the data or samples collected during routine care being reused for research purposes. The patient may object by writing a letter or email to the establishment's Data Protection Officer. Patients who object to the use of their data or samples for research purposes are excluded. None of the patients for who nasopharyngeal "*tube bottoms*" had been used raised any objections to their reuse.

The Senegalese specimens were collected from the Fatick region. This community, located 155 km south-east of the capital, Dakar, has an estimated population of around 48,000, spread over 30 villages covering an area of 230 km2. Samples were collected at four health posts, including namely the Diohine Privé, Diohine Public, Toucar, and Ngayokheme health posts. Nasopharyngeal swabs were taken from febrile patients (temperature ≥ 38°C) who consulted one of the four health posts in 2020, 2021, and 2022. As soon as the sample was taken, the swab was introduced into a Virocult tube containing a biological preservation liquid (Medical Wire, Corsham, UK). The samples were first stored at -20°C in the field, then transported in an electric cooler at -20°C from Niakhar to Dakar, where they were stored at -80°C, before being transported in a polystyrene box containing ice packs (temperature around 4°C) by air to Marseille (France), where they were stored at -80°C before analysis. The entire study was approved by Senegal's National Health Research Ethics Committee (declaration numbers #00087 and #00081). Each participant, or their legal guardian in the case of minors, also provided their written informed consent.

Nucleic acid extraction

Extraction of genetic material (DNA and RNA) from each sample was performed using a KingFisher system (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) with the MacheryNagel "NucleoMag" kit (Macherey-Nagel, Hoerdt, France) following the manufacturer's recommendations. A volume of 150 µL of nasopharyngeal sample was extracted and then eluted with 150 µL of elution buffer.

In-house multiplexed PCR tests

Primers and probes

All PCR systems that were multiplexed have already been published [16,18-35]. When planning a multiplex system, primers and probes should be tested in different combinations of fluorescence for probes and concentrations to achieve uniform performance. The complete list of primer sequences and fluorescent probes (FAM, VIC, and JUN) for all the microorganisms selected in this study, as well as the extract quality control (actin) and the final primer and probe concentrations used in PCR reactions are detailed in Supplementary Table 1.

Duplex and triplex PCR systems were designed to detect 23 microorganisms, including 18 viruses. Briefly, eleven systems, including nine duplexes (four targeting DNA microorganisms and five targeting RNA microorganisms), and two triplexes (targeting RNA microorganisms) were used. The combinations of duplex and triplex qPCR assays were as follows: (1) *Staphylococcus aureus* and human adenovirus (HAdV); (2) *Haemophilus influenzae* and *Streptococcus pyogenes*; (3) *Streptococcus pneumoniae* and *Mycoplasma pneumoniae*; (4) actin and human bocavirus (HBoV); (5) human metapneumovirus (hMPV) and human rhinovirus; (6) human parechovirus (HPeV) and respiratory syncytial virus (RSV); (7) human coronavirus OC43 (HCoV-OC43) and enterovirus; (8) human parainfluenza virus-2 (HPIV-2) and human parainfluenza virus-1 (HPIV-1); (9) human parainfluenza virus-3 (HPIV-3) and human parainfluenza virus-4 (HPIV-4); (10) human coronavirus HKU1 (HCoV-HKU1), human coronavirus 229E (HCoV-229E), and human coronavirus NL63 (HCoV-NL63); and (11) influenza A, influenza B, and SARS-CoV-2.

In-house multiplex DNA amplification assays

Each in-house multiplex DNA qPCR assay was performed in a final volume of 20 µL, including 5 µL of extract, 10 µL of 2x LightCycler 480 Probes Master (Roche Diagnostics, Meylan, France), each primer and probe at its specific concentration (Supplementary Table 1), and supplemented with water to reach the reaction volume. qPCR assays were performed on a LC480 thermal cycler (Roche).

Amplifications were performed using the following conditions: 95°C for five minutes followed by 40 cycles of denaturing at 95°C for three seconds, 60°C for 35 seconds, and finally a cooling step to 40°C for 40 seconds.

In-house multiplex RNA amplification assays

Each in-house multiplex qRT-PCR assay was performed in a final volume of 20 µL, including 5 µL of extract, 5 µL of 4x TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific, Grand Island, NY, USA), each primer and probe at its specific concentration (Supplementary Table 1), and supplemented with water to reach the reaction volume. qRT-PCR assays were performed on a LC480 thermal cycler (Roche) according to manufacturer's recommendations. Amplifications were performed using the following conditions: reverse transcription at 50°C for five minutes, and 95°C for 20 seconds, followed by 40 cycles of denaturing at 95°C for 15 seconds, 60°C for 60 seconds and, finally, a cooling step to 40°C for 40 seconds. For DNA and RNA amplifications assays, negative and positive controls were systematically used for each run to guarantee the reliability and validation of the analyses.

Evaluation of in-house multiplexed PCR tests

The results obtained with in-house multiplex PCR assays were systematically compared with those obtained using the same PCR systems but in simplex mode and with those obtained with a commercial system, using the FTD® Respiratory Pathogens 21 kit (Fast-Track Diagnostics, Esch-sur-Alzette, Luxembourg), used according to the manufacturer's recommendations. The performance of the different duplex and triplex PCR assays was thus evaluated in several ways. A first series of samples which were positive for the various microorganisms and human actin, as well as negative samples, were analysed in parallel by simplex PCR assay. A second series of samples which were positive for the various microorganisms and human actin, as well as negative samples, were analysed in parallel by the commercial FTD® kit. It was not possible to test the same samples with the PCR simplex assay and the FTD® commercial kit, as these were tube bottoms and quantities were insufficient to test everything in parallel. The cost of reagents and the duration of duplex and triplex qPCR assays were also estimated.

Statistical analysis

Categorical variables were reported as frequencies and percentages. Fisher's exact test and phi coefficient (Φ) were used to measure the strength of association between microorganisms. A two-sided P-value of less than .05 was considered statistically significant. Analyses were carried out using SAS 9.4 statistical software (SAS Institute, Cary, North Carolina, USA).

Results

Evaluation of duplex and triplex PCR assays

Overall, to evaluate duplex and triplex PCR assays, data obtained from 965 different DNA and RNA extracts including 408 samples which were positive for a targeted respiratory microorganism or for human actin were analysed.

Comparison with system using simplex qPCR

To compare duplex and triplex qPCR assays with simplex qPCR assays, 445 DNA/RNA extracts, including 203 positive samples, were analysed with the different strategies. Among the 445 DNA/RNA extracts analysed, here were 14 for *S. aureus* (five of which were positive), 14 for adenovirus (seven of which were positive), 13 for *H. influenzae* (six of which were positive), 13 for *S. pyogenes* (six of which were positive), 15 for *S. pneumoniae* (five of were positive), 15 for *M. pneumoniae* (eight of which were positive), 48 human actin (42 of which were positive), 48 for bocavirus (23 of which were positive), 16 for metapneumovirus (five of which were positive), 16 for rhinovirus (nine of which were positive), 17 for parechovirus (eight of which were positive), 17 for RSV (seven of which were positive), 14 for HCoV-OC43 (five of which were positive), 14 for enterovirus (seven of which were positive), 15 for HPIV-2 (seven of which were positive), 15 for HPIV-1 (seven of which were positive), 15 HPIV-3 (eight of which were positive), 15 for HPIV-4 (six of which were positive), 21 for HCoV-NL63 (eight of which were positive), 21 for HCoV-HKU1 (five of which were positive), 21 for HCoV-229 (six of which were positive), 16 for influenza A (five of which were positive), 16 for influenza B (four of which were positive), and 16 for SARS-CoV-2 (five of which were positive). The data obtained showed excellent sensitivity and specificity of the duplex and triplex qPCR assays, and are summarised in Supplementary Table 2. More precisely, all the results which were expected to be negative were indeed negative, and all the results which were expected to be positive were positive.

The Cycle Thresholds (CTs) were almost identical for most of the targets. For a few others, there were no more than two CTs differences between the different molecular assays.

Comparison with the FTD® Respiratory Pathogens 21 kit

To compare the duplex and triplex qPCR assays with a commercial kit (the FTD® Respiratory Pathogens 21 kit), 520 DNA/RNA extracts, including 205 positives, were analysed with the different strategies. Of the 520 DNA/RNA extracts analysed, there were 23 for *S. aureus* (including 11 positive), 23 for adenovirus (including nine positive), 24 for H. *influenzae* (including 16 positive), 24 for *S. pyogenes* (including six positive), 17 for *S. pneumoniae* (including seven positive), 17 for *M. pneumoniae* (including eight positive), 15 human actin (including five positive), 15 for bocavirus (including five positive), 23 for metapneumovirus (including nine positive), 23 for rhinovirus (including 12 positive), 23 for parechovirus (including five positive), 23 for RSV (including 17 positive), 19 for HCoV-OC43 (including seven positive), 19 for enterovirus (including ten positive), 18 for HPIV-2 (including six positive), 18 for HPIV-1 (including ten positive), 17 HPIV-3 (including six positive), 17 for HPIV-4 (including six positive), 22 for HCoV-NL63 (including eight positive), 22 for HCoV-HKU1 (including six positive), 22 for HCoV-229 (including six positive), 32 for influenza A (including 12 positive), 32 for influenza B (including seven positive), and 32 for SARS-CoV-2 (including 11 positive). The data obtained showed excellent sensitivity and specificity of the duplex and triplex qPCR assays, and are summarised in Supplementary Table 3. More precisely, all the results which were expected to be negative were indeed negative, and all the results which were expected to be positive were positive. The CTs were almost identical for most of the targets. For a few others, there were no more than two CT differences between the different molecular assays.

Cost and length of duplex and triplex qPCR analyses

The cost of analyses was estimated on the basis of the pathogens and reagent costs applied at Marseille University Hospitals (France) (Table 1). DNA/RNA extraction using the KingFisher device and the Macherey-Nagel "*NucleoMag*" kit was estimated at €2.79 excluding value-added tax (VAT) per sample. Each DNA duplex qPCR targeting two DNA pathogens was estimated at €0.35 excluding VAT. Each RNA duplex qPCR targeting two RNA pathogens was estimated at €1.28 excluding VAT.

In-house PCR assays[1]		FTD® Respiratory Pathogens 21 kit[2]		BioFire® Respiratory Panel 2. 1 plus (RP2.1 plus) kit[3]	
Targets	**Cost per sample**	**Targets**	**Cost per sample**	**Targets**	**Cost per sample**
23 pathogens	**€10.48 excl. VAT**	**21 pathogens**	**€33.75 excl. VAT**	**23 pathogens**	**€86.20 excl. VAT**
by combining all the	Extraction costs not included		Extraction costs not included		Including extraction costs
systems used	(+€ 2.79 = € 13.27 excl. VAT)		(+€ 2.79 = € 36.54 excl. VAT)		

[1]The in-house PCR assays is composed of four duplex DNA qPCR tests (€0.35 x 4 = €1.40 excl. VAT), five duplex RNA qPCR tests (€1.28 x 5 = €6.40) and two triplex RNA qPCR tests (€1.34 x 2 = €2.68 excl. VAT). [2]The cost of a FTD® Respiratory Pathogens 21 kit is €1080 excl. VAT (one kit allowing for the analysis of 32 samples). [3]The cost of a BioFire® Respiratory Panel 2. 1 plus (RP2.1 plus) kit is €2586 excl. VAT (one kit allowing for the analysis of 30 samples). Excl. VAT = excluding Value-Added Tax.

Table 1: Estimated costs of in-house multiplex PCR assays compared to commercial kits used in our clinical microbiology diagnostic laboratory.

The RNA triplex qPCR targeting three RNA pathogens was estimated at €1.34 excluding VAT. The lengths of analysis were estimated taking into account the various steps involved in extraction, amplification, and handling. Extraction time was 25 minutes. DNA qPCR time was one hour, and RNA qPCR time was one hour 25 minutes, with an additional ten minutes of handling. The estimated time for DNA qPCR was one hour 25 minutes, and two hours for RNA qPCR.

Senegalese patients and respiratory microorganisms

A total of 500 nasopharyngeal samples were analysed from 500 Senegalese patients. These 500 samples are part of a larger series of 2000 samples. Only a quarter of the samples had previously been analysed by a commercial kit, due to the excessive cost [36]. Of the 500 patients, 146 (29.2%) were aged between one month and five years, 154 (30.8%) between six and ten years, 111 (22.2%) between 11 and 20 years, and 89 (17.8%) were 21 years and over. The mean age of the patients was 12 years (youngest one month, oldest 73 years). Of these 500 samples, 261 (52.2%) were from women, 235 (47%) from men and this information was not specified for four patients (0.8%).

Prevalence of respiratory microorganisms

A respiratory microorganism was detected in at least 294 of the 500 nasopharyngeal samples (58.8%). Of these, 54.2% (271/500) were infected with at least one bacterium and 20.2% (101/500) were infected with a virus (Table 2). *S. pneumoniae* was the most prevalent bacterium detected (36.2%, 181/500), followed by *S. aureus* (21.6%, 108/500) and *H. influenzae* (20.2%, 101/500). Only four nasopharyngeal samples were positive for *S. pyogenes* (0.8%). No samples were positive for *M. pneumoniae*. Adenovirus was the most prevalent virus detected (5.2%, 26/500), followed by RSV (3.0%, 15/500), SARS-CoV-2 (2.2%, 11/500), rhinovirus (2.0%, 10/500) and influenza A virus (1.8%, 9/500) (Table 2). Enterovirus, HCoV-OC43, and human bocavirus were each detected in five patients (1.0%), HPIV-1 and influenza B virus each in four patients (0.8%), and HPIV-3 and HCoV-HKU1 each in three patients (0.6%). Human metapneumovirus was observed in two patients (0.4%). Finally, HPIV-2 and HCoV-NL63 were each detected in one patient (0.2%). No samples were positive for HPIV-4, parechovirus, or HCoV-229E.

	All (n=500)	**Patients from 1 month to 5 years (n=146)**	**Patients from 6 to 10 years (n=154)**	**Patients from 11 to 20 years (n=11)**	**Patients > 21 years (n=89)**
Microorganisms	**Number of positives (%)**				
At least one microorganism	294 (58.8%)	106 (72.6%)	100 (65%)	52 (46.8%)	36 (40.4%)
At least one bacterium	271 (54.2%)	101 (69.1%)	93 (60.3%)	54 (48.6%)	23 (25.8%)
At least one virus	101 (20.2%)	48 (32.9%)	32 (20.8%)	11 (9.9%)	10 (11.2%)
Bacteria					

Streptococcus pneumoniae	181 (36.2%)	78 (53.4%)	64 (41.6%)	30 (27.0%)	9 (10.1%)
Staphylococcus aureus	108 (21.6%)	24 (16.4%)	40 (26%)	32 (28.8%)	12 (13.4%)
Hemophilus influenzae	101 (20.2%)	54 (37.0%)	33 (21.4%)	12 (10.8%)	2 (2.2%)
Streptococcus pyogenes	4 (0.8%)	0	3 (2.0%)	1 (0.9%)	0
Mycoplasma pneumoniae	0	0	0	0	0
Viruses					
Adenovirus	26 (5.2%)	15 (10.2%)	8 (5.2%)	1 (0.9%)	2 (2.2%)
RSV	15 (3.0%)	7 (4.8%)	6 (3.9%)	2 (1.8%)	0
SARS-CoV-2	11 (2.2%)	4 (2.7%)	2 (1.3%)	2 (1.8%)	3 (3.4%)
Influenza A	9 (1.8%)	2 (1.3%)	4 (2.6%)	2 (1.8%)	1 (1.1%)
Influenza B	4 (0.8%)	0	3 (2.0%)	1 (0.9%)	0
Influenza A and B	13 (2.6%)	2 (1.3%)	7 (4.5%)	3 (2.7%)	1 (1.1%)
Rhinovirus	10 (2.0%)	7 (4.8%)	3 (2.0%)	0	0
Enterovirus	5 (1.0%)	5 (3.4%)	0	0	0
HPIV-1	4 (0.8%)	2 (1.3%)	2 (1.3%)	0	0
HPIV-2	1 (0.2%)	0	0	1 (0.9%)	0
HPIV-3	3 (0.6%)	1 (0.7%)	1 (0.6%)	0	1 (1.1%)
HPIV-4	0	0	0	0	0
HPIV 1, 2, 3, and 4	8 (1.6%)	3 (2.0%)	3 (2.0%)	1 (0.9%)	1 (1.1%)
HCoV-HKU1	3 (0.6%)	1 (0.7%)	1 (0.6%)	0	1 (1.1%)
HCoV-E229	0	0	0	0	0
HCoV-NL63	1 (0.2%)	0	1 (0.6%)	0	0
HCoV-OC43	5 (1.0%)	1 (0.7%)	0	2 (1.8%)	2 (2.2%)
All common coronavirus	9 (1.8%)	2 (1.3%)	2 (1.3%)	2 (1.8%)	3 (3.4%)
Metapneumovirus	2 (0.4%)	2 (1.3%)	0	0	0
Parechovirus	0	0	0	0	0
Bocavirus	0	0	0	0	0

Table 2: Prevalence of respiratory microorganisms detected using duplex and triplex qPCR assays in nasopharyngeal swabs from 500 febrile patients in the Niakhar area of Senegal.

Co-infection with respiratory microorganisms and their correlations

Co-infection was detected in 28.2% (141/500) of the nasopharyngeal samples (Table 3). Of the 141 co-infections, 82 (58.1%) involved two microorganisms. The combination of *S. pneumoniae* and *H. influenzae* was the most common (40.2%, 33/82), followed by *S. pneumoniae* and *S. aureus* (14.4%, 11/82), then *S. pneumoniae* and adenovirus (7.3%, 6/82) (Supplementary Table 4). Fifty-three out of 141 co-infections (37.6%) involved three microorganisms (Table 3). The three most frequent triple infections were as follows: (1) *S. pneumoniae*, *H. influenzae*, and *S. aureus* (26.4%, 14/53); (2) *S. pneumoniae*, *H. influenzae*, and adenovirus (15.1%, 8/53); and (3) *S. pneumoniae, H. influenzae*, and RSV (15.1%, 8/53) (Supplementary Table 4).

Number of respiratory microorganisms detected per sample	n (%)	Patients from 1 month to 5 years (n=146)	Patients from 6 to 10 years (n=154)	Patients from 11 to 20 years (n=11)	Patients > 21 years (n=89)
None	206 (41.2%)	40 (27.4%)	54 (35.0%)	52 (46.8%)	60 (67.4%)
One micro-organism	153 (30.6%)	38 (26.0%)	50 (32.5%)	40 (36.0%)	25 (28.0%)
Co-infections > two microorganisms	**141 (28.2%)**	**68 (46.6%)**	**50 (32.5%)**	**19 (17.1%)**	**4 (4.5%)**
Two micro-organisms	82 (16.4%)	38 (26.0%)	31 (20.1%)	10 (9.0%)	3 (3.4%)
Three micro-organisms	53 (10.6%)	28 (19.1%)	15 (9.7%)	9 (8.1%)	1 (1.1%)
Four micro-organisms	6 (1.2%)	2 (1.4%)	4 (2.6%)	0	0

Table 3: Number of respiratory microorganisms identified by nasopharyngeal sampling in 500 febrile patients from the Niakhar region of Senegal.

Finally, six co-infections out of 141 (4.2%) involved four microorganisms, as follows: (1) *S. pneumoniae, H. influenzae, S. aureus*, and adenovirus; (2) *S. pneumoniae, H. influenzae, S. aureus*, and HCoV-HKU1; (3) *S. pneumoniae, S. aureus, S. pyogenes*, and influenza A virus; (4) *S. pneumoniae*, *H. influenzae, S. aureus*, and influenza B virus; (5) *S. pneumoniae, H. influenzae*, metapneumovirus, and adenovirus; (6) *S. pneumoniae, H. influenzae, S. pyogenes*, and HCoV-NL63 (Supplementary Table 4).

The correlation analysis for all respiratory microorganisms reveals a statistically significant positive association between the presence of *H. influenzae* and *S. pneumoniae* (Φ=0.46, P<0.001) (Supplementary Table 5). Similarly, a strong positive correlation was observed between HCoV-NL63 and *S. pyogenes* (Φ=0.50, P<0.001). Moderate positive correlations were also identified between HPIV-1 and bocavirus (Φ=0.22, P<0.001) and between RSV and *S. pneumoniae* (Φ=0.21, P<0.001).

Seasonality of respiratory microorganisms

The prevalence of each respiratory microorganism by month and season is illustrated in Figures 1, 2, and 3. *S. pneumoniae, H. influenzae* and *S. aureus* can be observed all year round, but have higher peaks during the dry season (Figure 1). A peak in cases of SARS-CoV-2 infection was detected in January (Figure 2). While sporadic cases of influenza A virus infection are observed throughout the year, a major peak was observed in October (Figure 2). Cases of influenza B virus infection were observed exclusively in November and December, with a major peak in December. Rhinovirus was mainly observed during the rainy season (Figure 2). Bocavirus was exclusively observed during the dry season from April to May (Figure 3), whereas RSV was observed almost exclusively during the rainy season, with a peak in September (Figure 3). Adenovirus circulates year-round with a higher prevalence during the dry season, and a peak in March (Figure 3). Circulation of HCoV-OC43 was mainly observed during the rainy season (June, July, August, and October). Low circulation of HCoV-HKU1 was also noted during the rainy season (June and July) (Figure 2).

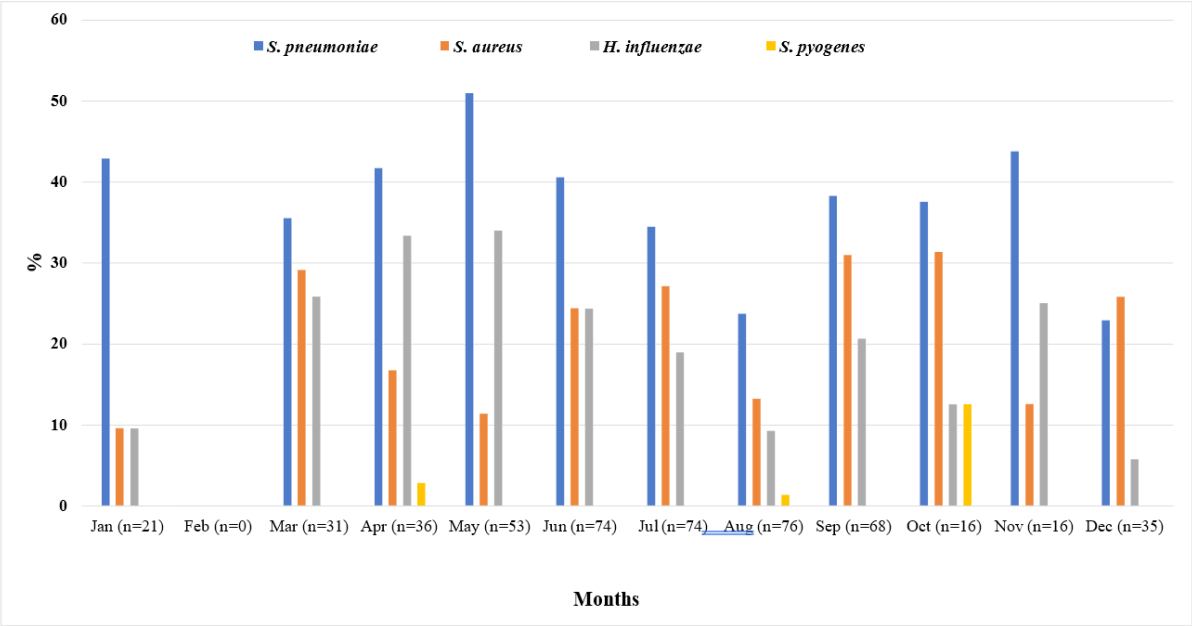

Figure 1: Seasonal prevalence of S. *pneumoniae*, S. *aureus*, *H. influenzae*, and S. *pyogenes* from nasopharyngeal swabs of febrile patients in the Niakhar region of rural Senegal (number of tested samples in parentheses).

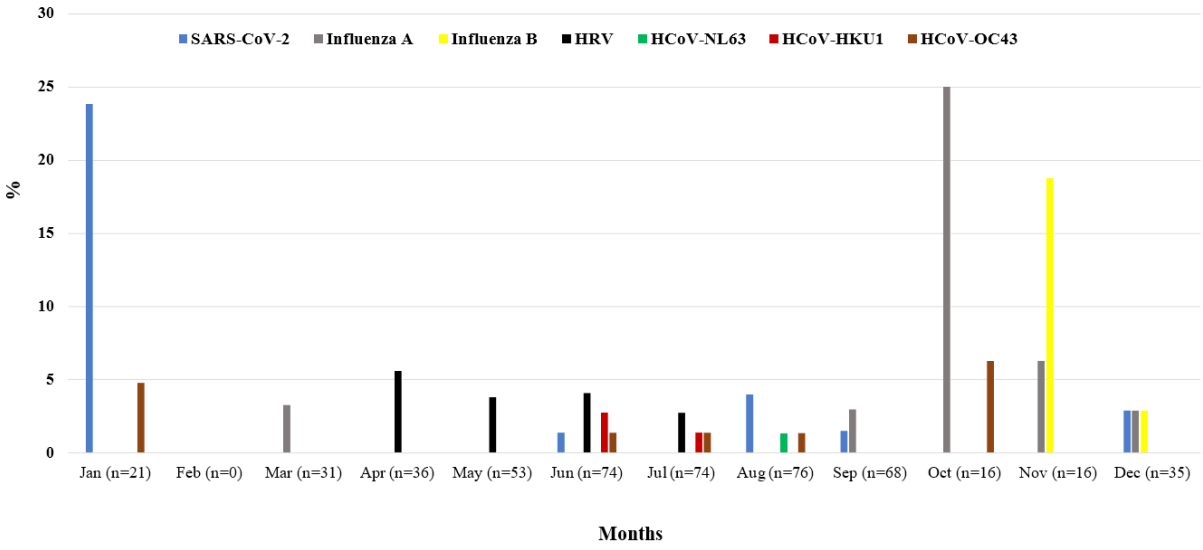

Figure 2: Seasonal prevalence of respiratory viruses, including SARS-CoV-2, influenza A, influenza B, HRV, HCoV-NL63, HCoV-HKU1, and HCoV-OC43 from nasopharyngeal swabs of febrile patients in the Niakhar region of rural Senegal (number of tested samples in parentheses).

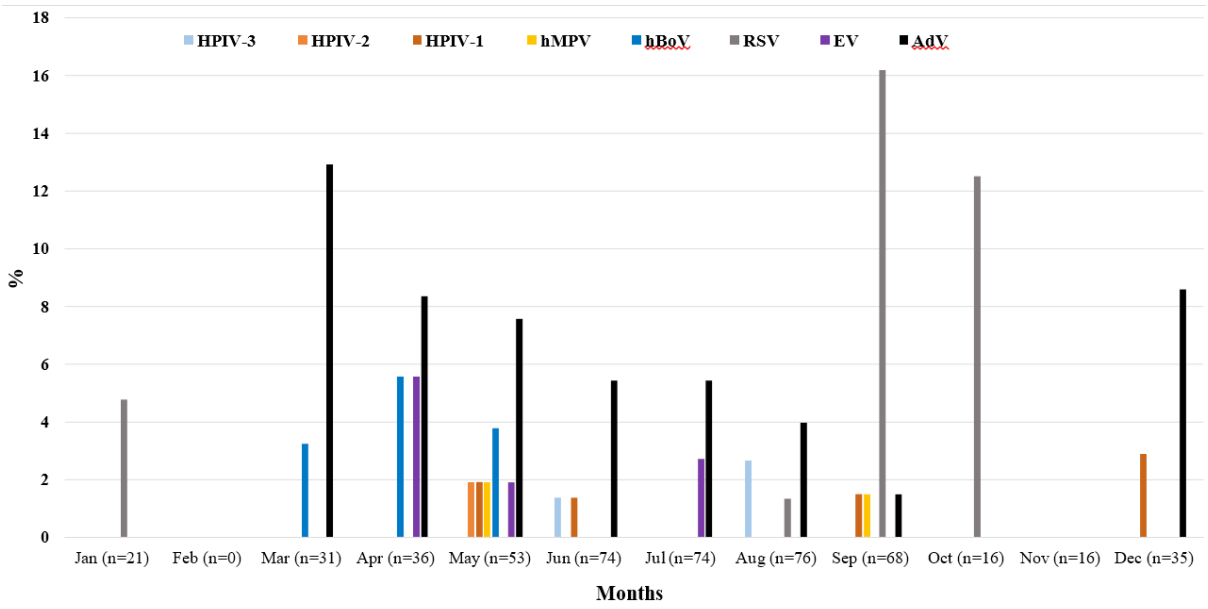

Figure 3: Seasonal prevalence of respiratory viruses, including HPIV-3, HPIV-2, HPIV-1, hMPV, hBoV, RSV, enterovirus (EV), and adenovirus (AdV) from nasopharyngeal swabs in 500 febrile patients from the Niakhar area in rural Senegal (number of samples tested in brackets).

Discussion

The range of potential pathogens involved in acute respiratory infections is vast [37]. Clinical symptoms do not allow for a specific diagnosis but, at best, presumptions, and co-infections can have a potential impact on clinical manifestations [37]. Historically, establishing a microbiological diagnosis required a combination of microscopy and culture [39]. The first tools which were developed for the rapid diagnosis of acute respiratory infections were based on rapid antigenic kits, providing results in less than 30 minutes. However, given the scale of false negatives and the lack of sensitivity of these tests, more sensitive molecular tools were then developed and deployed [37,38]. Initially, based on simplex microorganism-specific PCR assays, large syndromic panels capable of simultaneously detecting several microorganisms, mainly respiratory viruses, gained great popularity among medical biology laboratories and physicians alike [37].

The theoretical advantage of this large-scale molecular strategy is to be able to make an aetiological diagnosis to best adapt the patient's care, bearing in mind that there is currently no real treatment available for viral infections, apart from influenza and RSV. The question of whether or not to prescribe antibiotics, in a global context of the fight against antibiotic resistance, is often raised [39].

In any case, one of the undeniable benefits of these multiplexed tests are infection control and prevention practices. However, while the benefits of multiplex testing appear to be particularly significant for epidemiological studies and clinical research, its impact on patient care remains under debate [39]. Indeed, the place of this strategy raises a number of questions. First of all, there is the question of the real medical benefit, depending on: (1) the patient's age (the role it can play in paediatrics, geriatrics, and adult patients); (2) the absence or presence of immunodepression, and its type and severity; (3) the patient's clinical severity; (4) associated comorbidities; (5) the place of consultation (community facility, emergency ward, hospital wards, intensive care units etc.); (6) the need to hospitalise the patient; and (7) the rapid availability of results, so that management can be adapted as quickly as possible [39-41]. As well as the medical relevance of the strategy, the cost/benefit ratio is also under discussion. Finally, in theory, diagnosis can also reduce uncertainty for physicians and anxiety for patients and their families [39,42]. The PCR kits which are available on the market are still far too expensive for some countries, not to mention the fact that they require the use of special equipment, which means additional costs. Application of the multiplex PCR method in a routine diagnostic context, implementation time, time-to-result, costs, and outcomes are also very important parameters to consider [43].

Overall, multiplexed approaches differ according to several criteria, including: (1) the degree of multiplexing, with the ability to detect viruses as well as bacteria; (2) the complexity of the method, ranging from medium to high; (3) the throughput, which can be low to high; (4) the use or non-use of unit tests; and (5) the execution time, which can range from 45 minutes to almost six hours. However, not all bacteria which play an important role in determining respiratory infections are systematically included. Furthermore, some microorganisms included in the main syndromic panels are rarely detected. It is important to take into account the local epidemiology, the ecosystem, as well as the seasonality of certain infections. It would not appear to be necessary to search for all microorganisms at the same time, but rather to target respiratory microorganisms commonly identified in epidemiological studies, in order to be able to adapt targets according to seasonality and epidemic context. In turn, this would make it possible to offer much less costly molecular tests rather than using expensive syndromic panels to detect pathogens that are too varied. The technological characteristics of molecular tests must reflect the realities of the field (simple, rapid, sensitive, and specific tests aimed at a wide range of targets, at an acceptable cost) and the needs and expectations of physicians.

Duplex and triplex in-house PCR systems were designed by combining existing simplex systems with a variety of the type of fluorophores for the detection of respiratory pathogens, including viruses and bacteria responsible for respiratory infections. The analytical performance (sensitivity and specificity) of multiplexed molecular tests showed excellent concordance with simplex molecular tests as well with the commercial FTD® Respiratory Pathogens 21 kit. These in-house systems are easy to use to prepare mixtures and interpret results. They require only standard PCR equipment, but with two channels of fluorescence reading for duplex and three channels of fluorescence reading for triplex. The analysis time for in-house systems is between 85 and 120 minutes, depending on the type of pathogen, double the time for the BioFire® Respiratory Panel 2. 1 plus (RP2.1 plus) kit, but approximately equivalent to FTD® Respiratory Pathogens 21 kit. However, the cost analysis clearly shows that in-house systems are less expensive than commercial kits, mainly the BioFire® kit, although that kit requires less technician handling time, as it requires special equipment.

One of the advantages of duplex and or triplex systems is that they can be tailored to individual patients, unlike current platforms which offer few or no options for selecting the targeted microorganisms to be tested. Depending on the fluorochromes used (such as FAM, VIC, JUN, etc.), different systems can be combined as required. Moreover, the number of pathogens to be screened can be adapted according to local epidemiology, patient age, time of year, geographic region, and host immunocompetence, further reducing the cost of analysis with in-house systems.

Based on our study and previous Senegalese studies, *S. pneumoniae* is the most prevalent microorganism detected in nasopharyngeal swabs from febrile patients, reaching up to 85.9% among children under the age of one with an acute respiratory infection [36,44,45]. Overall, pneumococcus remains the main pathogen responsible for bacterial pneumonia, despite the fact that vaccination coverage with the third dose of pneumococcal conjugate vaccine in one-year-old children in Senegal has been equal to or greater than 80% since 2014, according to the World Health Organization (https://data.who.int/indicators/i/D45F91C, accessed on 17 October 2024). The high prevalence of *S. pneumoniae* could be linked to vaccine failure, with, among other causes, the circulation of serotypes which are usually not present. As with *H. influenzae*, another frequently observed bacterium, the circulation of *S. pneumoniae* is recorded all year round but is at a higher level during the dry season (from November to May) in the Niakhar area [36].

Adenovirus was the most frequently detected virus in our study. Other Senegalese studies have also shown that its prevalence is high, reaching up to 50% among children under five years of age with upper and lower respiratory infections [36]. Its temporal distribution confirms previous results, showing year-round circulation, with a fluctuating prevalence but no evidence of seasonality [46]. In our study, RSV, a major cause of hospitalisation in young children, is the most frequently detected virus after adenovirus. RSV also displays a clear seasonal pattern, with circulation observed almost exclusively during the rainy season (June to October), as reported in previous Senegalese studies [36].

SARS-CoV-2 was the most prevalent coronavirus in our study. It was mainly observed during the month of January 2022, corresponding to the spread of the fourth wave of COVID-19 in Senegal [47]. The prevalence of other viruses detected in our study was either between 2% and 1% (rhinovirus, influenza A, enterovirus, HCoV-OC43, bocavirus), or less than 1% (HPIV-1, influenza B, HPIV-3, HCoV-HKU1, metapneumovirus, and HCoV-NL63, HPIV-2). Overall, influenza A is still frequently detected in nasopharyngeal swabs in Senegal, with a higher prevalence during the rainy season [36].

Finally, as previously reported, co-infections with several respiratory microorganisms were also observed [36], most of them involving *S. pneumoniae* and *H. influenzae* associated with *S. aureus* or various other viruses.

Conclusion

The use of in-house duplex and triplex qPCR assays is efficient, enabling the simultaneous and rapid detection of several microorganisms responsible for respiratory infections. They are also less costly than commercial kits, which represent a considerable

financial burden for countries with limited resources. Their use can be modular, targeting pathogens according to local epidemiology, age, or season. In the future, Senegalese epidemiological data will enable us to target the most frequent microorganisms, namely *S. pneumoniae*, *H. influenza*e, adenovirus, RSV, SARS-CoV-2, and influenza viruses for diagnosis, in order to further limit costs.

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Ethical Considerations

The study was conducted in accordance with the Declaration of Helsinki, and was approved by the National Ethics Committee for Health Research of Senegal (NECHR) (Dakar, Senegal) under agreement nos. 00087 MSAS/DGS/DS/CNERS and 00081 MSAS/DGS/DS/CNERS.

Conflict of Interest

The authors have no conflicts of interest to declare. Funding sources had no role in the design and conduct of the study; collection, management, analysis and interpretation of the data; and preparation, review, or approval of the manuscript.

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Author Contributions

FF, GD, HB, OM, and CS conceived the study. DN, GD, HB and MS collected the samples and compiled the clinical data. DN, AJSN and MBB performed the analyses. DN, SC, AJSN, MBB, SE, PEF, OM, CS, and FF interpreted data. FF, GD, HB, SE, OM, PEF, CS, and FF reviewed the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

Data generated during this study are available from the corresponding author on reasonable request.

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