Epidemiology of acute respiratory viral infections in children in Vientiane, Lao People's Democratic Republic

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Funding information
Luxembourg Institute of Health; Ministry of Foreign and European Affairs, Luxembourg, Grant/Award Number: PaReCIDS II

Abstract
Respiratory infections are one of the most frequent reasons for medical consultations in children. In low resource settings such as in Lao People’s Democratic Republic, knowledge gaps and the dearth of laboratory capacity to support differential diagnosis may contribute to antibiotic overuse. We studied the etiology, temporal trends, and genetic diversity of viral respiratory infections in children to provide evidence for prevention and treatment guidelines. From September 2014 to October 2015, throat swabs and nasopharyngeal aspirates from 445 children under 10 years old with symptoms of acute respiratory infection were collected at the Children Hospital in Vientiane. Rapid antigen tests were performed for influenza A and B and respiratory syncytial virus. Real-time reverse-transcription polymerase chain reactions (RT-PCRs) were performed to detect 16 viruses. Influenza infections were detected with a higher sensitivity using PCR than with the rapid antigen test. By RT-PCR screening, at least one pathogen could be identified for 71.7% of cases. Human rhinoviruses were most frequently detected (29.9%), followed by influenza A and B viruses combined (15.9%). We identify and discuss the seasonality of some of the infections. Altogether these data provide a detailed characterization of respiratory pathogens in Lao children and we provide recommendations for vaccination and further studies.

Keywords
acute respiratory infections, children, human metapneumovirus, influenza virus, Lao PDR, respiratory syncytial virus

This paper is dedicated to the memory of Dr. Somxay Billamay, who passed away in January 2021.

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1 | INTRODUCTION

In children, respiratory tract infections are one of the most common reasons for seeking medical care. In Lao People's Democratic Republic (PDR), consultations for influenza-like illness (ILI) represented 10% of outpatient consultations in Vientiane between 2008 and 2010. The incidence of ILI was estimated at 10.7 episodes per 100 person years in the Vientiane metropolitan area between 2015 and 2016, with the highest rates in children below 15 years old. The etiology of respiratory tract infections is very diverse. A high proportion of viral infections present with mild symptoms, which may worsen due to bacteria superinfections. Measures to mitigate bacteria coinfections or gaps in differential diagnosis often lead to empirical antibiotic treatment. Antibiotic prescription levels in primary care settings in low and middle-income countries are often high, and are influenced by staff training level. However, innate and adaptive immune responses to pathogenic microorganisms are influenced by commensal bacteria, which are present in site-specific communities on mucosal surfaces. Resident non-pathogenic bacteria of the airways augment the immunity against viral infections. Therefore, empirical antibiotic treatment of respiratory infections without a laboratory confirmation may disrupt the balance necessary for effective virus clearance. In addition, unnecessary antibiotic administration increases the risk of emergence and spread of antibiotic resistance.

In Lao PDR, knowledge gaps in respiratory tract infection diagnosis and management exist. In a previous study, nearly 30% of the medical doctors questioned thought that unnecessary antibiotic treatment was harmless. In addition, weak laboratory capacity to substantiate differential diagnosis can lead to unnecessary antibiotic prescription, often driven by patient demand or antibiotic availability rather than necessity. A surge of patients with ILI was reported between May and July 2008 in Vientiane but this did not coincide with increased transmission of influenza cases. Previous studies in the country were confined to a limited number of pathogens, mainly influenza viruses, with almost half of all patients left without etiological diagnosis. This, combined with the age-specific pathogen susceptibility, warrants a broadening of respiratory surveillance programs. This study therefore aimed at investigating the etiology of respiratory infections in children, a population with higher susceptibility and disease severity. The study aimed at including a larger panel of viral agents than previous studies, to provide better evidence on disease etiology to local medical doctors.

2 | MATERIALS AND METHODS

2.1 | Ethics approval and consent to participate

The study was approved by the Lao National Ethics Committee for Health Research (No. 027/NIOPH/NECHR). Clinical samples and data were collected after obtaining informed consent from the patient’s parents or legal guardians.

2.2 | Inclusion criteria, sample and data collection

The inclusion criteria were: age below 10-year old, presenting to the Children Hospital in Vientiane with symptoms of acute respiratory infection; informed consent obtained. Throat swabs and nasopharyngeal aspirates were collected by trained hospital personnel. A questionnaire capturing demographic and clinical data was administered by health care personnel.

2.3 | Virus detection and characterization

Rapid antigen (Ag) tests for influenza A/B/A (H1N1) and respiratory syncytial virus (RSV) (SD Bioline) were performed on the nasopharyngeal aspirates on the day of collection according to manufacturer’s instructions. The remaining material was transported to the Institut Pasteur du Laos (IPL) where nucleic acid purification with TRIzol LS (Life Technologies) and Pure Link Viral RNA/DNA Mini Kits (Life Technologies) was performed. RNA and DNA were eluted in RNase free water and stored at −80°C and −20°C, respectively.

Influenza A virus detection was performed on throat swabs by real-time reverse transcription polymerase chain reaction (RT-PCR) with MP-39-67For, MP-183-153Rev primers, and MP-96-75ProbeAs probe and QuantiTect Probe RT-PCR Kit (Qiagen). Detection of influenza B and C virus, RSV, human metapneumovirus (hMPV), parainfluenzaviruses 1-4 (PIV1-4), human coronaviruses 229E, OC43, HKU1 and NL63 (hCoV-229E, -OC43, -HKU1, -NL63) and human rhinoviruses (hRV) was performed separately on both throat swabs and nasopharyngeal aspirates in four conventional multiplex PCR assays using Qiagen OneStep RT-PCR Kit (Qiagen). Human adenovirus (hAdV) and human Bocavirus (hBoV) were detected in a pool of equal volume of DNA purified from both types of samples by a fifth multiplex PCR using TaqMan Universal PCR Master Mix (Life Technologies). Influenza A virus subtyping was performed by two real-time RT-PCRs detecting pandemic H1N1 strains (A/H1N1pdm) or seasonal H3N2 strains (A/H3N2). RSV positive samples were identified as RSV-A or RSV-B by a duplex real-time RT-PCR with QuantiTect Probe RT-PCR Kit.

Complete glycoprotein (G) sequences of RSV strains and partial fusion (F) and glycoprotein (G) sequences of hMPV were amplified using previously published primer pairs. PCR product purification and sequencing were performed as described before. The nucleotide substitution model that best fitted the data was determined with MEGA6. Genetic distances and phylogenetic analyses were then calculated with the best model using the Neighbor-joining method and 500 bootstrap replicates in MEGA6.
2.4 | Data analyses

Statistical analyses were performed using SigmaPlot version 12.5. McNemar's test with Yates correction for continuity was used for comparing detection rates between swab types or between RT-PCR and rapid tests. Mann–Whitney rank-sum tests were used to assess the effect of age on overall positivity rate (≥1 virus detected), on the detection of single or mixed infections, on the positivity rate for each virus, on antibiotic prescription, and to assess the effect of delay between onset of symptoms and sampling on the outcome of rapid tests. A one-tailed t test was applied to assess the effect of influenza C values on rapid test outcomes. Odds ratios were calculated to describe the risk associated with positivity for each viral species and hospital admission. Odds ratios were also calculated to evaluate antibiotic prescription with regard to in- or outpatient treatment.

Based on the sample collection date, cases were classified as occurring during the rainy or dry seasons. In Lao PDR, the rainy season, typically taking place between mid-October and mid-May of the following year, is marked by frequent and heavy rain with high humidity. Rainy seasons alternate with dry seasons characterized by low humidity and high temperatures that occurs from mid-May to mid-October. Z-scores were calculated to assess the seasonality of virus occurrence in the dry or rainy season.

3 | RESULTS

3.1 | Demographic data

From September 2014 to August 2015, 445 children (median age 18.6 months; Table 1) were recruited at the Children's Hospital in Vientiane. Patients presented with fever (n = 390) and/or cough (n = 418) and/or nasal congestion (n = 403; Table 1). The majority (80.9%) were outpatients while 19.1% (85 of 445) were admitted to the hospital. Throat swabs and nasopharyngeal aspirates were collected from all patients (n = 445).

3.2 | Prevalence of respiratory viruses by PCR

Overall, at least one virus was detected by RT-PCR in 71.7% (319 of 445; Table 1) of the patients when combining results from both types of swabs. hRV was the most frequently detected pathogen (133 of 445, 29.9%) followed by influenza A virus (54 of 445, 12.1%; Figure 1). All other viruses were detected at lower levels (2.7%–8.1%) and PIV2, PIV4, and the 4 hCoVs were detected only sporadically; in less than 2% of the patients (Figure 1). Although detection rates were the same or higher in nasopharyngeal aspirates compared to throat swabs for most pathogens (10 of 13), a statistically significant difference was only observed for RSV (p = .039; Figure 1B).

Mixed infections were detected in 13.5% (60 of 445; Table 1) of the patients, with a majority of dual viral infections (57 of 60, 95.0%) compared to triple viral co-infections (3 of 60, 5.0%). Nineteen pathogen combinations were found, most combinations (13 of 19, 64.8%) being observed only in 1 or 2 patients. hRV was involved in 75.0% (45 of 60) of the mixed infections and most frequent combinations involved hRV together with hAdV (15 of 60, 25.0%) or together with influenza C virus (10 of 60, 16.7%). No statistically significant difference between the age of children with single or mixed infection was observed (p = .542).

3.3 | Comparison of rapid antigen detection and PCR detection methods

Detection rates of influenza A and B viruses by rapid tests (38 of 432, 8.8%; 13 samples were not tested by rapid tests) were significantly lower than cumulative detection by the two PCRs (71 of 432, 16.4%; p < .001). These results suggest a high rate of false negatives by rapid antigen tests. For the patients with influenza A and B infections confirmed by PCR, the delay between the reported date of symptom onset and sampling did not affect the outcome of detection by rapid test (p = .348). For influenza A,

| Table 1 | Demographic, clinical, and virus detection rate data of the 445 patients enrolled |
|----------------------|------------------|
| Variable             | No. of patients (%) |
| Gender               |                  |
| Female               | 190 (42.7)       |
| Male                 | 255 (57.3)       |
| Age group            |                  |
| 0–6 months           | 55 (12.4)        |
| 7–12 months          | 94 (21.1)        |
| 13–24 months         | 130 (29.2)       |
| 25–60 months         | 131 (29.4)       |
| 5–10 years           | 35 (7.9)         |
| Patient admission    |                  |
| Outpatient           | 360 (80.9)       |
| Inpatient            | 85 (19.1)        |
| Symptoms             |                  |
| Fever                | 390 (87.6)       |
| Cough                | 418 (93.9)       |
| Nasal congestion     | 403 (90.6)       |
| Detection of viral infections |                |
| Positive for at least 1 virus | 319 (71.7)   |
| Single viral infection | 259 (58.2)   |
| Mixed viral infections | 60 (13.5) |

*aPositivity by real-time reverse-transcription polymerase chain reaction (RT-PCR) results from throat swabs and nasopharyngeal aspirates combined.

*bIncludes the number of cases where two or three viruses were detected.
samples with higher C\textsubscript{t} values were less likely to be positive by rapid test (\textit{p} = .026). No statistically significant difference was observed when comparing RSV detection by rapid test (37 of 436, 10.8%) or conventional PCR (28 of 436, 6.4%; \textit{p} = .814). Nine RSV rapid test positive specimens were PCR negative, which may indicate either a lack in sensitivity of the conventional PCR and/or false positives by rapid tests.

3.4 | Age distribution, disease severity, and antibiotic treatment

The overall positivity rate (≥1 virus detected) and presence of mixed infections were not associated with age (\textit{p} = .556 and \textit{p} = .542, respectively). Patients with influenza A (\textit{p} = .017), influenza B (\textit{p} = .004) or hAdV (\textit{p} = .026) infections were significantly
older. Patients with RSV ($p = .010$) or hRV ($p = .046$) infections were significantly younger.

Only RSV infection was associated with an increased risk of hospital admission (OR = 2.527, $p = .030$). Antibiotic prescription was reported for 23.1% (103 of 445) children. Inpatients were more likely to receive antibiotics than outpatients (30 of 85, 35.3% vs. 73 of 360, 20.3%; odd ratio = 2.144, $p = .005$), but there was no relationship between age and antibiotic prescription ($p = .439$).

3.5 | Virus typing and seasonal trends

Influenza A was detected in 12.1% of patients (54 of 445) and the strains were characterized as A/H1N1 pdm (16 of 54, 29.6%) and A/H3N2 (32 of 54, 59.3%). Influenza B was detected in 3.8% (17 of 445) of the patients. Influenza A/H1N1 pdm, A/H3N2, and influenza B co-circulated between week 36 (September 2014) and week 13 (March 2015); weeks 26 to 39 (June to September 2015) were dominated by A/H3N2 with sporadic detections of influenza B viruses (Figure 2).

RSV was exclusively detected in September 2014 and 2015 within the rainy season ($p < .001$). RSV strains were typed as RSV-A (17 of 31, 54.8%) and RSV-B (12 of 31, 38.7%) by PCR. All RSV-A strains belonged to the ON1 genotype (Figure S1). The genetic similarity between Lao strains ranged from 98.2% to 100% over the complete genome sequence. All RSV-B sequences clustered within the BA9 genotype and were distinct from BA9 strains that were circulating in the country in 2010.21 The THB/CB1 genotype previously identified in Lao PDR in 201021 was not observed in this study.

hMPV was detected almost all year round without any temporal variation ($p = .738$). hMPV strains were typed as genotype A2b (12 of 17, 70.6%), B1 (1 of 17, 5.9%) and B2 (4 of 17, 23.5%) based on partial F gene sequences (Figures 3A and S2A) and confirmed based on partial G gene sequences (Figure S2B). No temporal variation of strain circulation was observed and A2b, B1, and B2 strains were co-circulating: for instance, all three genotypes were detected in August 2015 (Figures 3A and S2).

PIV3 ($p < .001$) and hCoV-HKU1 ($p = .002$) were detected more frequently during the dry season. No seasonal differences were observed for the other viruses ($p = .103$ to 0.995).

4 | DISCUSSION

In Lao PDR, limited disease surveillance data prompted us to investigate and characterize a wide range of respiratory pathogens. Here we show that respiratory viral infections occur throughout the year in Lao PDR, with hRV being the most frequently detected virus (29.9% overall and 75.0% of co-infections; Figure 1). High frequencies of hRV infections were also reported in other studies carried out in Lao PDR, regardless of the population investigated.3,11,12,22 Two studies found that hRV was the most frequently detected virus in all age groups in patients with ILI or acute lower respiratory infections.11,12 hRV was also the second most frequent virus reported, after RSV, in children with severe respiratory acute infection.22 Although other studies carried out elsewhere have shown that hRV can be found in 10%–33% of asymptomatic children23–25 and has typically been associated with
upper respiratory tract infections, its implication in more severe
diseases such as bronchiolitis, pneumonia, or asthma exacerbation
has also been suggested. Co-infections tend to increase illness
duration: hospital stay for children with hRV-RSV co-infections has
been shown to be significantly longer than children infected with
RSV or hRV alone. In addition, hRV-C may be more virulent than
hRV-A and -B. Therefore, the importance of hRV should not be
underestimated and further investigations and molecular char-
acterization of hRV strains in mild and severe respiratory infections
in Lao PDR are warranted.

Temporal trends of influenza A and B viruses observed during
our study were concordant with a peak of A/H3N2 circulation in
September 2015 reported during ILI community surveillance in
nearly 1000 selected households in Vientiane. It also aligns with
the sentinel surveillance in Lao PDR (FluNet), which found A/H1N1 pdm,
A/H3N2, and influenza B co-circulating during January–June 2015,
followed by a dominance of A/H3N2 during the second half of the
year. Thus, our data and other surveillance activities revealed two
influenza seasons of equal intensities in 2015. This situation con-
trasts with the overall reported pattern of influenza circulation with
low but steady levels of transmission from January to August follow-
ed by a period of increased transmission between September and
December (Figure S3). Current recommendations for seasonal
influenza vaccination in April–May in Lao PDR seem adequate to
protect the population during the main influenza period. Risk groups
grouped for influenza vaccination in the country include pregnant
women, the elderly, healthcare personnel, and people with co-
morbidities, but currently not children. Influenza vaccination in
children older than 6 months is recommended in certain coun-
tries. This is not only beneficial to reduce disease burden, espe-
cially in younger children at higher risk of severe complications,
but also contributes to herd immunity, and less transmission in the
community. In addition, the non-negligible proportion of cases
occurring outside the main influenza period (45% on average,
2011–2019), coupled with unusual years such as 2015, requires
further long-term monitoring in the country and brings into question
the existence of a seasonal pattern of influenza transmission in
Lao PDR.

Our study also confirms the seasonality of RSV circulation in Lao
PDR, with increased incidence in the rainy season. However,
we did not detect RSV cases outside September 2014 and 2015. This
contrasts with previous studies suggesting that RSV is present year-
round with a surge of cases during the rainy season. Younger
children had an increased chance of being positive for RSV and had
an increased risk of hospital admission, again highlighting the con-
tribution of RSV to respiratory disease severity in young children.

hMPV, discovered only two decades ago, shares clinical and epide-
miological characteristics with RSV. It has been shown that most
children are infected with hMPV before 5 years of age. hMPV in-
fecions lead to a wide range of clinical symptoms including frequent
bronchiolitis and pneumonia, with a peak of hospitalization for chil-
dren aged 6–12 months. However, no association with age or
hospital admission was observed in our study. Five main genotypes
have been described so far, often co-circulating. Indeed, our study
provides the first molecular characterization of hMPV in Lao PDR
and shows co-circulation of 3 genotypes. The predominant genotype
has been shown to change over time and is influenced by genotype-
specific levels of immunity in the community, virus evolution and
escape and differential replication advantage rather than a dif-
ference in virulence.

An analysis of 30 years of influenza surveillance data in France
highlighted the existence of a group of seasons showing similar
transmission patterns, in addition to single unusual seasons of
transmission. Temporal divergences were also associated with dif-
ferent spatial dissemination patterns between regions. Therefore,
in our setting, patient recruitment in a wider network of study sites
over a larger timespan is warranted to overcome the spatiotemporal
limitations of this study where children from only one hospital were
recruited over the course of one year. Enhanced country-wide
longitudinal surveillance will allow the identification of long-term seasonal trends of virus circulation in Lao PDR, both at the viral
species and genotypes/subtypes/serotypes levels. A more intensive
sampling comparing in- and outpatients will also be needed to fur-
ther understand the burden of viruses such as hRV, RSV, or hMPV.

In our study, we combined results from both nasopharyngeal
aspirates and throat swabs. This approach allowed for improved
sensitivity for identifying the etiological agents of respiratory infec-
tions in a pediatric population. Others have also found that multiple
sampling in an adult population with lower respiratory tract mani-
festations revealed an improved sensitivity for virus detection.
Nasopharyngeal aspirates provided a significantly increased sensi-
tivity for detection only for RSV. This contrasts with a comparison
of nasal, throat or nasopharyngeal swabs for the detection of RSV in
hospitalized children in Lao PDR that showed no statistical differ-
ence between all three sampling materials. However, viral loads in
throat swabs in that study were lower than in nasal or nasophar-
yngeal swabs, which can influence detection rates by conventional
PCR. Nevertheless, this approach is cumbersome in low resource
settings. As the comparison of detection rates between the two
types of samples revealed no statistical difference for the vast ma-
majority of viruses studied, surveillance of viral upper respiratory tract
infections using a sampling technique requiring less professional
skills such as throat or nasal swab collection, compared to naso-
pharyngeal aspirates, can be envisaged to cover larger areas of
Lao PDR.

Antibiotics were prescribed to 20.3% of outpatients in the Children
Hospital in Vientiane Capital. This contrasts with overall high levels
of 52% of antibiotic prescription in primary care settings in low and middle-
income countries. In rural areas of China, antibiotics were prescribed in
37%–55% of consultations for upper respiratory tract infections, while
doctor training and healthcare level influenced prescription levels.
The apparent low antibiotic prescription rate in our study likely results
from higher expertise and awareness in the capital. Indeed, Quet et al.
showed a difference in perception of antibiotic overuse by medical
doctors in the Vientiane capital compared to Luang Prabang and
Khammouane provinces (82.8% vs. 61.4% and 56.9%, respectively).
Easier access to information such as through the Internet or training likely contributes to improved knowledge.

With an infection characterization rate of more than 70%, our data provide a detailed overview of the dynamics of respiratory pathogens in the study population. This information will be useful to raise awareness among local medical practitioners and may provide guidance in planning vaccination campaigns in the country. Given the importance of influenza A/B infections, seasonal influenza vaccination for young children in Lao PDR could help decrease the annual burden. However, other childhood vaccinations may have a higher priority. We also show permanent co-circulation of several respiratory viruses, contributing to ILI cases all year round. This highlights the need to take them into account for differential diagnosis of respiratory infections outside the influenza season. Coronavirus were among the least frequently detected viruses, but their importance has changed with the emergence and worldwide spread of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The risk of future seasonal circulation of SARS-CoV-2 now requires the integration of this new pathogen in the respiratory disease differential diagnosis. The broad spectrum of disease severity induced by SARS-CoV-2 infections further justifies a more systematic laboratory diagnostic approach towards respiratory viruses and the improvement of already existing vaccinations against respiratory pathogens such as influenza to decrease the overall burden of respiratory (co-) infections.

DATA AVAILABILITY STATEMENT
Nucleotide sequences associated with this study are available on the European Nucleotide Archive under the study accession number PRJEB42813 and sequence accession numbers LR999699-LR999752. The data that support the findings of this study are available upon reasonable request to the corresponding author, after obtaining permission from the National Ethics Committee for Health Research (NECHR) in the Lao PDR. The data are not publicly available due to privacy or ethical restrictions.

ACKNOWLEDGMENTS
We would like to thank the participants and the staff at the Children’s Hospital, Vientiane. We also thank the staff and administration at Institut Pasteur du Laos for their support. The study was funded by the Luxembourg Ministry of Foreign Affairs and by the Luxembourg Institute of Health, “PaReCIDS II” grant.

CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

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Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Snoeck CJ, Evdokimov K, Xaydalasouk K, et al. Epidemiology of acute respiratory viral infections in children in Vientiane, Lao People’s Democratic Republic. J Med Virol. 2021;93:4748-4755. https://doi.org/10.1002/jmv.27004