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Sebastien Kenmoe, Marie-Astrid Vernet, Véronique Beng Penlap, Astrid Vabret, Richard Njouom. Phylogenetic variability of Human Metapneumovirus in patients with acute respiratory infections in Cameroon, 2011–2014. Journal of Infection and Public Health, In press, 10.1016/j.jiph.2019.08.018 . hal-02366670

HAL Id: hal-02366670

https://normandie-univ.hal.science/hal-02366670

Submitted on 19 Nov 2019

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Journal of Infection and Public Health xxx (2019) xxx–xxx

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: http://www.elsevier.com/locate/jiph

Phylogenetic variability of Human Metapneumovirus in patients with acute respiratory infections in Cameroon, 2011–2014

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ARTICLE INFO

Article history:
Received 10 November 2018
Accepted 26 August 2019

Keywords:
Acute respiratory infections
Human Metapneumovirus
Genetic variability
Sub-Saharan Africa
Cameroon

ABSTRACT

Background: Identified in 2001, Human Metapneumovirus (HMPV) is a Pneumovirus associated with acute lower and upper respiratory infections in all age groups and especially in newborns, elderly and immunocompromised subjects. Data are still limited in sub-Saharan African countries genetic characterization of this respiratory virus. This study reports the genetic variability of HMPV strains in Cameroonian children for 3 consecutive epidemic seasons (September 2011–October 2014).
Methods: A prospective surveillance was conducted to identify inpatient and outpatient children less than 15 years with respiratory symptoms ≤5 days. The nasopharyngeal samples were tested for HMPV using a multiplex polymerase chain reaction. Viral distribution and demographic data were analyzed statistically. Positive samples for HMPV were amplified by semi-nested polymerize chain reaction and then partially sequenced at the G gene. Phylogenetic analyzes were performed on the partial nucleotide and protein sequences of the G gene.
Results: From September 2011 to October 2014, 822 children under 15 years were enrolled in the study. HMPV was identified in each of 3.9% (32/822) of children. HMPV were detected throughout the year. HMPV-A (73.3%; 11/15) was predominant compared to HMPV-B (26.7; 4/15). Cameroonian HMPV strains are grouped among the members of genotype A2b (for HMPV-A), B1 and B2 (for HMPV-B).
Conclusion: This study suggests that about 4% of ARI recorded in children in Cameroon are caused by HMPV. The present study is also the first report on the genetic variability of the G gene of HMPV strains in the region. Although this work partially fills gaps for some information, additional studies are required to clarify the molecular epidemiology and evolutionary pattern of HMPV in sub-Saharan Africa in general and more particularly in Cameroon.

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Introduction

Human Metapneumovirus (HMPV), first identified in the Netherlands in 2001 [1], is an important causative agent of acute respiratory infections worldwide in susceptible individuals, particularly children, adults and immunocompromised patients [2]. This virus accounts for 6.24% (95% CI 5.25–7.30) of hospitalized patients with acute respiratory tract infections [3]. Since its identification, HMPV has been identified in many countries across five continents, including Africa [4–9]. This virus causes annual epidemics in late winter and early spring in temperate regions, while in subtropical regions it peaks in spring and summer [4–6,10]. HMPV is an enveloped virus with a genome consisting of a single-stranded and non-segmented RNA molecule approximately 13 kb in length [1]. This genome contains eight genes that code for 9 different proteins in the order 3′-NPMF-M2-1/M2-2-SH-GL-5′. With the exception of the M2 gene, which has two overlapping open reading frames, all other genes have a single open reading frame. The viral enve-

Abbreviations: HMPV, Human Metapneumovirus.

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https://doi.org/10.1016/j.jiph.2019.08.018
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lope contains three transmembrane surface glycoproteins in the form of 13–17 nm projections, the highly glycosylated attachment glycoprotein G, the fusion protein F and the small hydrophobic protein SH [11]. F and G proteins promote binding, membrane fusion and viral penetration into the host cell cytoplasm [12]. These two proteins are also the main antigenic determinants that stimulate the neutralizing antibody response. Protein F is relatively conserved while G has the greatest genetic and antigenic variability [5,13,14]. Taxonomically, the HMPV belongs to the *Pneumoviridae* family and *Pneumovirinae* sub-family. Genetically, its closest relative is the Avian Pneumovirus Type C with whom HMPV shares the closest relationship with respect to sequence identity and gene constellation. Based on the analysis of the entire genome or other genes, HMPV was divided into two distinct genotypes A and B and subdivided into four subgenotypes A1, A2, B1 and B2 [1,13,15]. Subgenotype A2 was further classified into two categories A2a and A2b [15]. Although this classification is consistent independently of the gene studied, the great genetic and antigenic variability between them is mainly found on the surface gene G [5,13–15], whereas the other genes are much more conserved [16,17]. The dynamics and history of HMPV evolution have been studied thanks to the advent of a large number of HMPV sequences in databases. Unlike other paramyxoviruses, the four HMPV genetic genotypes have been preserved over time because the gene sequences, like the G and F genes, do not display significant progressive genetic drift [18]. In this respect, the G gene has strongly conserved motifs in its ectodomain. Multiple studies have helped to show that the 4 HMPV subgroups can cocirculate simultaneously with the predominance of one of the subgroups and alternation of the majority genotype from year to year [4,10,13–15]. It is also well understood that the different lineages can circulate simultaneously in the same place during a given season [5,19–23]. The importance of this highly variable and complex circulation model remains, however, still largely uncertain in sub-Saharan Africa. Despite its importance as a respiratory pathogen, information on molecular characterization of HMPV from sub-Saharan African countries remains limited [9]. Repeated HMPV infections during life are common and there is currently no effective vaccine [24,25]. The possibility of reinfection with HMPV could be a challenge in developing a future HMPV vaccine. This reinfection could be either because of incomplete immunity or genetic changes of the virus. For the development of vaccines, a thorough understanding of the genetic and antigenic heterogeneity of HMPV transmembrane glycoproteins from all origins is therefore necessary. In Cameroon, we have previously reported HMPV circulation among 4% hospitalized children and 5% ambulatory patients with acute respiratory infections [26,27]. In this study, we examine the extent of genetic variability of the HMPV G gene in Yaounde, Cameroon for three consecutive years.

Material and methods

Design of the study

This work is a cross-sectional study that was conducted for 3 years from September 2011 to October 2014. The participants were recruited from the Centre Hospitalier d'Essos, Yaoundé, Cameroon. Yaounde is one of the most populous city of the Cameroonian state with about 2.8 million inhabitants. This city is located in the Central region and is covered by an equatorial climate with alternating two dry seasons (mid-November to early March and mid-June to early September) and two rainy seasons (early March to mid-June and early September to mid-November).

Inclusion criteria

The study was done by a non-probability sampling and a consecutive recruitment. The subjects included in the survey were children under 15 years who were outpatients or hospitalized and consulted for acute respiratory tract infections. Ambulatory participants were eligible if they had a sudden-onset fever with a temperature >38 °C less than 5 days with cough and/or sore throat. Inpatients consisted of individuals with the above criteria in addition to hospitalization. Children whose parents or guardians did not give their consent for the study were not included.

Ethical considerations

Samples were submitted to the Pasteur Center of Cameroon as part of the IMMI project Ïnstitute of Microbiology and Infectious Diseases. The procedures of the original study (IMMI project) were evaluated and approved by the National Research Ethics Committee and the Ministry of Public Health of Cameroon (authorization N° 121/CNE/SE/2011). Written informed consent was obtained from the parents or guardians of the children recruited in the study. A numerical code has been assigned to all included individuals and no confidential participant data has been disclosed in this work.

HMPV detection

A nasopharyngeal swab was collected either at the time of consultation or after hospitalization. The sample was placed in a tube containing 1 mL of viral transport medium, transported in coolers in the laboratory, and stored at −80 °C until analysis. The viral RNA was extracted using QIAamp Viral RNA Mini kit and QIAamp DNA Mini kit and according to the manufacturer's recommendations (Qiagen, Hilden, Germany). The extracted RNA were screened for HMPV using a commercially Respiratory Multi Well System r-gene™ (bioMerieux, Lyon, France) as previously described [28].

HMPV genotyping

The HMPV-positive samples were amplified by a conventional RT-PCR targeting G gene [13,14]. The RT-PCR reaction was carried out using the SuperScript® III One-Step RT-PCR System kit according to the manufacturer's instructions (Thermo Fisher Scientific, Carlsbad, CA, USA). Briefly, 5 μL of RNA was added to a 45 μL PCR reaction mixture containing 16 μL of water, 25 μL of 2X reaction mix, 1 μL of forward primer HMPVG_Fwd (5′-GAGAACATTCGRRCRATAGAYATG-3′) at 10 μM, 1 μL of reverse primer HMPVG_Rev (5′-AGATAGACATTRACAGTGGATTCA-3′) at 10 μM, and 2 μL of Platinum® Taq DNA Polymerase. The HMPVG_Fwd primer corresponds to position 6262–6285 and the HMPVG_Rev primer to nucleotides 7181–7204 of the reference sequence 001 (Genbank number: AF371337). The thermal profile used consisted of a reverse transcription at 55 °C for 30 min, a denaturation step at 94 °C for 2 min; followed by 40 cycles at 94 °C for 15 s, 55 °C for 30 s and 68 °C for one minute; and a final extension at 68 °C for 5 min. Genotyping PCR amplification products were sequenced bidirectionally using BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Foster City, CA, USA). The primers used for sequencing were the same as those used for genotyping RT-PCR.

Phylogenetic analysis

The forward and reverse sequences were edited and assembled into a single consensus. Peaks of poor quality at the sequences extremities were removed. The predicted nucleotide sequences were translated into protein sequence. The consensus sequences

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Table 1
General data of the study participants.

Characteristics		Total	Human metapneumovirus	
		No (822)	Negative 790 (96.1)	Positive 32 (3.9)
Gender				
	Male	437 (53.2)	423 (53.5)	14 (43.8)
	Female	384 (46.7)	366 (46.3)	18 (56.3)
	NA	1 (0.1)	1 (0.1)	
	p-Value			0.3
Age				
	[0–6] months	102 (12.5)	99 (12.5)	3 (9.4)
	[6–24] months	413 (50.6)	394 (49.9)	19 (59.4)
	[2–5] years	210 (25.7)	203 (25.7)	7 (21.9)
	[5–15] years	92 (11.3)	90 (11.4)	2 (6.3)
	NA	5 (0.6)	4 (0.5)	1 (3.1)
	p-Value			0.6
Patients				
	Inpatients	436 (53)	419 (53.0)	17 (53.1)
	Outpatients	386 (47)	371 (47.0)	15 (46.9)
	p-Value			1
Symptoms				
	Cough	691 (84.1)	664 (83.6)	27 (96.4)
	Rhinorrhea	660 (80.3)	637 (80.2)	23 (82.1)
	Fatigue	475 (57.8)	456 (57.4)	19 (67.9)
	Wheezing	295 (35.9)	281 (35.4)	14 (50)
	Breathlessness	170 (20.7)	161 (20.3)	9 (32.1)
	Diarrhea	168 (20.4)	161 (20.3)	7 (25)
	Vomiting	252 (30.7)	243 (30.6)	9 (32.1)
	Cutaneous rash	58 (7.1)	54 (6.8)	4 (14.3)
	Conjunctivitis	85 (10.3)	82 (10.3)	3 (10.7)
	Sore throat	140 (17.0)	137 (17.3)	3 (10.7)
	Headache	102 (12.4)	100 (12.6)	2 (7.1)
	Arthralgia	47 (5.7)	45 (5.7)	2 (7.1)
	Myalgia	24 (2.9)	23 (2.9)	1 (3.6)
	Earache	32 (3.9)	31 (3.9)	1 (3.6)

NA: not available. Data are number (%).

of this study as well as reference sequences from other countries around the world were aligned using the CLUSTAL W algorithm [29]. The comparison of similarity proportions was performed between our isolates and the HMPV- A2 reference strains (Genbank No. AY296021), HMPV- B1 (Genbank No. AY296034) and HMPV-B2 (Genbank No. AY296040). The phylogenetic tree was constructed using the Maximum Likelihood method and under the best model of evolution that was Tamura Nei with a Gamma distribution. The robustness of the tree was evaluated with 1000 resampling bootstrap. Potentially *N*- and *O*-glycosylated sites were predicted using NetNGlyc version 1.0 and NetOGlyc version 4.0 online servers respectively [30]. Sequences were assembled and edited using the EDITSEQ program in the Seqman™ II Lasergene software (DNA, Madison, WI, USA) and phylogenetic analyzes were performed using the MEGA Version 6 software [31]. The nucleotide sequences of this study were submitted to GenBank and recorded with accession numbers MK050259 to MK050273.

Statistical analysis

The data was analyzed using the R software version 3.4.1. Quantitative variables were presented as median and interquartile range (IQR). Categorical variables were expressed in numbers and proportions. The analysis assessed the potential associated risk factors (sex, age, and severity) for HMPV infections using the Pearson's Chi-square independence test with a significance level of less than 5%.

Results

Study population

A total of 822 children were recruited between September 2011 and October 2014. The median age of study participants was 19 months IQR [9–36]. The age of the patients ranged from 1 month to 15 years. Of the 822 children recruited, 12.5% were less than 6 months, 50.6% between 6 and 24 months, 25.7% between 2 and 5 years, and 11.3% between 5 and 15 years. The study included 437 (53.2%) boys and 384 (46.7%) girls, a sex ratio of 1.1 (437/384). Of the children, 436 (53%) were hospitalized while 386 (47%) were ambulatory.

Viral detection

HMPV was detected in 32 (3.9%, 95% CI 2.6–5.4%) of the 822 analyzed samples. The most common symptoms were cough, rhinorrhea, fatigue and wheezing (Table 1). Sex, age, and inpatient/outpatient status of children were not associated with HMPV infections. In 2012 and 2013 HMPV was detected almost throughout the year while no cases in 2011 and two sporadic cases in 2014 were detected.

HMPV genotyping

To differentiate the HMPV-positive samples, an 870 nucleotide fragment of the G gene was amplified for 15/30 (50%) tested samples. Half of the samples could not be amplified. The viral load of the unamplified samples was significantly lower than that of the amplified ones (33.3; IQR [31.7–35.1] vs 23.9; IQR [21.5–27.6]; $p < 0.01$). The partial G genes of all amplified HMPV were sequenced successfully.

Molecular epidemiology

Groups A (11) and B (4) of the HMPV were detected in 2012 and 2013 with a predominance of Group A in 2013 (10/12, 81.8%). Sporadic cases of subgroup HMPV-A2b (1), B2 (2) were detected in 2012. The year 2013 revealed one case of HMPV-B1 and one case of HMPV-B2. Cameroonian HMPV isolates, based on 467 bp of the G

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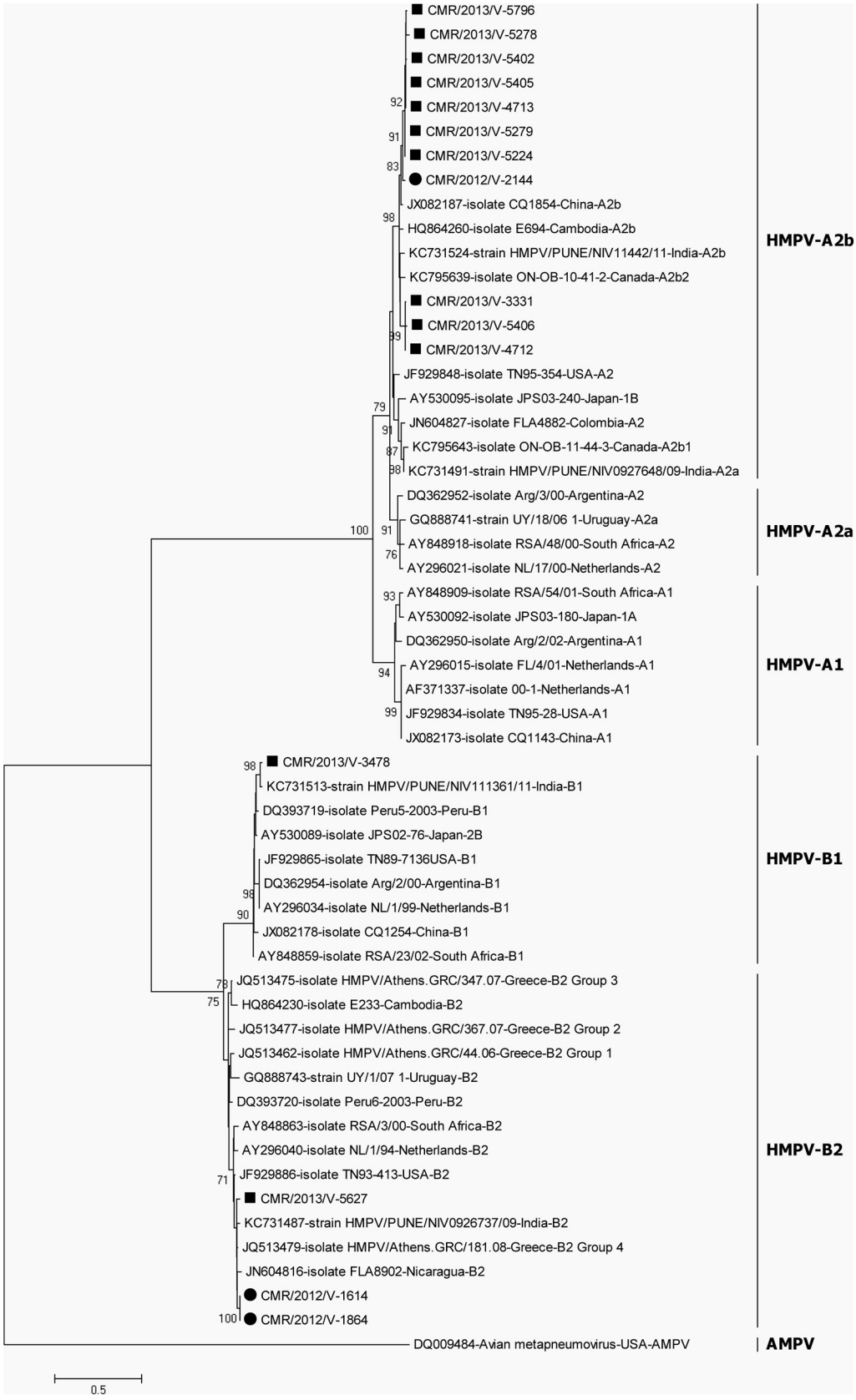


Fig. 1. Phylogenetic tree of HMPV-A and HMPV-B strains detected in Yaoundé, Cameroon from 2011 to 2013.
Multiple sequence alignment was performed with Clustal W. The rooted tree was generated based on G gene nucleotide sequences (467 nucleotides of position 6397–6864 of reference sequence 00-1 Genbank number AF371337) using the maximum likelihood method and the best model evolution (Tamura Nei) with a discrete gamma distribution in Mega 6. The numbers on the nodes of the branches are determined values for resampling *bootstrap* after 1000 iterations. Only values greater than or equal to 70% are presented. The reference sequences of different continents, obtained on Genbank, are identified from the left to the right with the access number, the name of the strain, the origin country and the genotype attributed by the author. The strains of the current study are designated from left to right by CMR (Cameroon), the year of detection and the laboratory number. Cameroon sequences are shown with (●) and (■) for the 2012 and 2013 sequences, respectively.

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gene, were grouped into three monophyletic branches, HMPV-A2b (11), B1 (1) and B2 (3) with reference strains from other countries (Fig. 1). No specific branch of time or space was observed.

Nucleotide and protein polymorphism

The description of the amino acid sequences deduced from the Cameroonian HMPV-A2b, HMPV-B1 and HMPV-B2 strains was carried out with reference to the partial sequence of the G genes of the NL/17/00 prototypes (AY296021, 190 residues: positions 31–221), NL/1/99 (AY296034, 209 residues: positions 25–233) and NL/1/94 (AY296040, 218 residues: positions 25–243), respectively. The G genes of the Cameroonian HMPV-A2b, HMPV-B1 and HMPV-B2 viruses had divergence rates ranging from 12.3 to 15%; 6.1%; and 6.2 to 6.8% at the nucleotide level and 23.4 to 26.9%; 15.5%; and 10.2 to 12.5% at the amino acid level, respectively. Transitions were most common than transversions for all HMPV Cameroonian sequences with a transition/transversion ratio of 7, 25, and 7.8 for HMPV-A2b, HMPV-B1 and HMPV-B2, respectively. The partial G gene sequences of the HMPV-A2b, HMPV-B1 and HMPV-B2 strains were aligned with sequences from other countries of the world and reference sequences (Supplementary Fig. 1A–C).

The Cameroon strains of genotype A2b, B1 and B2 had a length of 190, 208 and 217 amino acids, respectively. S90N, I151R and N155S substitutions were shared by all Cameroonian HMPV-A2b sequences and sequences from other countries. The substitutions A99T, Y133H, Y197N and R198G were shared by all Cameroonian HMPV-B2 sequences and sequences from other countries. An insertion of 4 amino acids was observed at position 160 of the HMPV-B2 genotype.

Glycosylation profile

Potential *N*-glycosylation sites were observed on the G protein sequences of the study (Supplementary Fig. 1A–C). An amino acid sequence of subgroup HMPV-A2b had a potential *N*-glycosylation site at position 52-54. Three potential *N*-glycosylation sites at positions 101–103, 169–171 and 188–190 were identified on the HMPV-B1 sequence of this study. For the G proteins of the HMPV-B2 subgroup, three potential N-glycosylation sites were identified, amino acids 68-60, 180–182 and 183-185. Mutations in the sequences of this study also led to gains or losses of potential *N*-glycosylation sites. In total, three additional *N*-glycosylation sites were present on some Cameroonian strains HMPV-A2b amino acids 105–107 (1/10), 131–133 (3/10) and 140–142 (3/10). The sequences of the study had lost potential *N*-glycosylation sites at amino acid positions 88–90 and 101–103 for HMPV-A2b and 181–183 for HMPV-B1. The number of serine and threonine residues, probably *O*-glycosylated ranged from 52 to 60, 59 and 63 to 64 for the HMPV-A2b, B1 and B2 sequences of the study respectively.

Discussion

During this study, we determined the epidemiology and carried out for the first time the molecular characterization of the nucleotide sequence and amino acid predicted of the partial G gene of HMPV isolated in nasopharyngeal swab of Cameroonian children from September 2011 to October 2014 at Yaoundé, Capital of Cameroon.

HMPV was identified in 3.9% of the samples tested. This frequency of detection corresponds to those we previously reported in Cameroon and those reported by other authors in Africa [7,9,26,27]. As in previous reports, there was no difference in infection rates between age groups ($p = 0.6$) [32]. Indeed, studies have shown that infections occur in all age groups probably due to partial immune protection or the acquisition of new genotypes [9,33]. Unlike the study by Wang et al. where the majority of positive HMPV participants were hospitalized, there was no difference between HMPV infection and hospitalization in this study [4]. In temperate regions, HMPV infections are distributed mainly in late winter and early spring [6,10]. Some studies, however, have shown that HMPV can be detected throughout the year [34]. To create more confusion some studies have shown that HMPV also circulates in summer [4,5]. Long-term studies have also shown a pattern of biennial circulation with low and high positive rates in odd and even years [6]. In addition, the peak of HMPV activity has been shown to vary by year in some studies [5]. There is very little data on the seasonality of HMPV in the tropical countries. For this study we are unable to clearly argue on HMPV seasonality because of the very low number of positive samples detected. However, in agreement with other tropical countries, the peaks of circulation were observed during the rainy season (September to December) [14]. Therefore, to elucidate the circulation pattern of HMPV in Cameroon, further, longer-term studies are warranted. As reported in other studies [8,9,20,33], 50% (15/30) of the HMPV positives were not amplified by RT-PCR for sequencing. This result could be due to a low viral load in unamplified samples. Indeed the viral load the unamplified samples were significantly lower than that of the amplified ones. Previous data has shown that two or more subgroups of HMPV can co-circulate in regions where HMPV is endemic [6,14,19–23,35]. Phylogenetic analysis of the partial G gene sequences in this study demonstrates the cocirculation in Cameroon of the two HMPV-A and B groups divided into three subgroups A2b, B1 and B2 during the study period. These three genotypes have also been reported in studies in Kenya and South Africa [13,9]. Similar to the previous studies [14], the HMPV-A1 genotype that were detected during the years 2000–2003 [13,16], were not identified in this study. The HMPV-A group is predominant in the HMPV-B group in most studies [36,37]. Similarly to these studies, of the 15 HMPV isolates of this study, 11 (73.3%) were grouped as HMPV-A (A2b sub group) and 4 (26.7%) strains were grouped as HMPV-B (3 B2 and 1 B1). As in the current study, HMPV-A group, subgroup A2b was more frequently detected in other studies [36,38]. Phylogenetic analysis have shown that subgroups of the HMPV virus are neither temporally nor spatially limited and are randomly distributed over the years and in different countries [39]. The data also showed that HMPV subgroups co-circulate with replacement of dominant subgroups every 1–3 years [6]. This alternate in the model of domination in HMPV groups may be due to the development of community immunity in response to dominant group from year to year. This complex pattern of HMPV circulation poses a real challenge for the development of a future vaccine. Very few studies have reported the distribution of HMPV subgroups in sub-Saharan Africa [9,22]. In this study, HMPV-A2b (1) and HMPV-B2 (2) were identified in 2012. In addition to one HMPV-B2 detected in 2013, a high increase in HMPV-A2b (10) and a case of HMPV-B1 have been highlighted. We must be cautious when interpreting on the variations of HMPV subgroups in Cameroon. Indeed, although three subgroups of HMPV were detected in this study, only 50% (15/30) of HMPV-positive samples were characterized from unrepresentative sampling. Therefore, it is impossible to say whether this would represent the pattern of alternation or predominance of HMPV subgroups in Cameroon during the study period. It is therefore necessary to conduct long-term studies in Cameroon in order to clarify the HMPV circulation pattern. As observed in previous studies [19], the G proteins of Cameroon subgroup B2 viruses had a length different from the reference strain. This longer length was attributed to an insertion of 12 additional nucleotides in the Cameroonian G sequences. The mutations S90N, I151R and N155S were present in all Cameroonian sequences, as observed in previous studies [13,14]. As previously reported [14,18,19], N52 glycosylation sites

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for HMPV-A2b (4/10); N101, N169 and N188 for HMPV-B1; N58, N180 and N183 for HMPV-B2 were observed in the Cameroonian sequences. Compared to the reference sequence (NL/17/00) [40], the Cameroonian sequences lost the N-glycosylation sites N88 and N101 and acquired 3 additional sites N105 (1/11), N131 (3/11) and N140 (3/10). In addition, the B1 sequence of this study lost the N181 *N*-glycosylation site observed in the reference strain [40].

Our study is, however, have several limitations. The collection of clinical data, the potential risk factors (malnutrition, immunodepression, congestion), and the outcome of included patients were not exhaustive. As a result, assessment of the burden, risk factors and pathogenicity of HMPV infections in Cameroon remains incomplete.

Conclusion

In conclusion, this is the first study in Cameroon to characterize HMPV-A and B in hospitalized and ambulatory pediatric patients for a 3 year consecutive period. In addition, the results acquired in the current study also provide for the first time data on HMPV genotypes detected in Cameroon. Genetic characterization of G glycoprotein, the main target of many vaccines under development, indicated that three HMPV subgroups (A2, B1 and B2) were identified during the study. This study highlights the importance of implementing other large-scale studies based on the general population in resource-limited countries like Cameroon where data are still very scarce.

Availability of data and material

The nucleotide sequences of this study were submitted to Genbank and registered with the accession numbers MK050259 to MK050273.

Funding

This work was funded by the U.S. Department of Health and Human Services (DHHS) grant number 6 DESP060001-01-01 via the International Network of Pasteur Institutes and the 'Institut de Microbiologie et de Maladies Infectieuses' (IMMI) in France.

Competing interests

None.

Author's contributions

N.J. designed the study; K.S. and V.M-A. carried out and interpreted experiments; K.S. conducted phylogenetic analyses. K.S. wrote the first draft of the paper; All authors reviewed the manuscript critically and approved of the final manuscript as submitted.

Consent for publication

Not applicable.

Acknowledgement

Not applicable.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jiph.2019.08.018.

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