

Original Article

## High genotypic diversity of Rhinoviruses obtained from Tunisian children with severe acute respiratory infection

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### Abstract

**Introduction:** Rhinoviruses (HRV) are among the leading causes of Severe Acute Respiratory Infection (SARI). Their burden and genetic diversity vary from one region to another and little is known in Northern African regions. This study describes epidemiological patterns and genotypic diversity of HRV in SARI cases during a two and half year's study, in Northern Tunisia.

**Methodology:** A total of 271 SARI cases, admitted into the Pediatric Intensive Care Unit of Bechir Hamza Children's Hospital in Tunis, were collected between September 2015 and December 2017. The investigation concerned 104 samples positive for HRV and/or HEV (Human Enterovirus) obtained among these cases. Specific HRV and HEV detections were assessed by real-time PCRs. The HRV molecular typing was based on the VP4-VP2 genomic region analyses.

**Results:** Among the viral SARI cases, 33.5% and 12.3% were positive for HRV and HEV respectively. Molecular investigations showed high prevalence of HRV-A (63.3%) followed by HRV-C (30.6%) and HRV-B (6.1%) and high genotypic diversity with 27 types. HRV cases were mostly detected in toddlers younger than 6 months. A total of 16 cases (28%) were found with bacterial and/or viral co-infection. HRV-C infection and HRV-A with bacterial co-infection were associated with complicated infection. Some of the detected types showed a continuous circulation or turnover during an extended period. HRV-A101 and HRV-C45 were the most frequently detected types.

**Conclusions:** This study revealed, for the first time, the high HRV diversity in Tunisia, a North-African region. Specific phylogenetic investigations may help to evaluate their diversity and to trace their spread and epidemiological origin.

**Key words:** Rhinovirus; incidence; type; Tunisia; SARI.

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### Introduction

Severe Acute Respiratory Infection (SARI) is one of the leading causes of morbidity and mortality in neonates and children under 5 years of age, worldwide causing approximately 4.2 million deaths annually [1]. In more than 80% of cases, a variety of respiratory viruses are implicated [2]. *Human Rhinoviruses* (HRV) are among the most prevalent agents in SARI cases. Nevertheless, in the Eastern Mediterranean regions, genetic diversity remains poorly defined especially in North African regions [3].

HRV are small non-enveloped, positive-sense RNA viruses that are now classified within the *Enterovirus* genus of the family *Picornaviridae* [4]. They are known

to be a common cause of upper respiratory diseases [5] and also considered among the leading causes of SARI, especially in younger children [6]. Clinical manifestations may vary from bronchiolitis and wheezing illnesses to pneumonia, and exacerbation of asthma [7-11]. In addition, early childhood HRV infection may cause airway damage leading to subsequent asthma development [12]. Similarly, to other *Picornaviridae* members, HRV were characterized by a wide genetic divergence. They include three species A, B and-C, subdivided into 169 types [4]. HRV-A was the most diverse, totaling 80 types, while the HRV-B includes 32 types. HRV-C was discovered in 2006, with the improvement of molecular

methods [13] and is subdivided into 57 types. The type identification is based on the discriminatory potential of specific genomic regions. The VP4/VP2 coding region is generally used, giving the ease of genomic amplification and the high discriminatory potential. The full VP1 genomic region is also recommended for type confirmation [14-16].

HRV species are globally distributed and in all age groups. Nevertheless, the main epidemiological and molecular data available describes HRV infections in Asian, European and North American regions and little is known in African and Middle Eastern regions, especially for circulating types [14,16]. In Tunisia, a northern African region, available data concerns only epidemiology and clinical characteristics of HRV infections, in restricted districts without any precision of circulating types [17,18]. This study aims to describe epidemiological characteristics and circulating types of HRV during a two- and half-year surveillance study on children with Severe Acute Respiratory Infection (SARI) in all northern Tunisian regions.

## Methodology

### *Ethics statement*

This study was approved by the local Ethics Committee of Bechir Hamza Children's Hospital of Tunis, Tunisia. It was performed in accordance with ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This study used samples obtained for diagnostic purposes on routine laboratory testing from a panel of respiratory viruses. The specimens were used after de-identification of the samples with respect to patient anonymity.

### *Study population and sampling*

A total of 271 nasopharyngeal aspirates was obtained between September 2015 and November 2017 from pediatric patients with SARI admitted to the Pediatric Intensive Care Unit (PICU) of Bechir Hamza Children's Hospital in Tunis (BHC Hospital), Tunisia. BHC Hospital is the one pediatric hospital in Tunisia which enrolls patients from Tunis (the capital), all northern districts (n = 11) and also complicated cases from other Tunisian regional hospitals. SARI cases were selected according to the WHO revised clinical case definition as all acute respiratory illness cases with a history of fever or measured fever of  $\geq 38^{\circ}\text{C}$  and cough, with onset within the past 10 days, requiring hospitalization [19].

Once collected, samples were submitted to the laboratory of Microbiology at BHC hospital and to the

laboratory of Virology at Charles Nicolle Hospital for bacteriological and virologic diagnosis, respectively. Virologic aliquots were transported in viral transport medium (Universal Transport Medium; COPAN Diagnostics Inc., Murrieta, CA) and at a temperature of  $4^{\circ}\text{C}$  to the laboratory. Aliquots of samples were also stored at  $-80^{\circ}\text{C}$  for additional research purposes such as molecular investigations.

For each sample, demographic and clinical data were collected. The report form included questions on gender, age, general demographic information, clinical signs and health status.

Bacterial diagnosis included microscopy direct examination and culture according to conventional methods.

The virology diagnosis included molecular detection of Influenza A and B viruses, Parainfluenza viruses; Respiratory Syncytial Virus (RSV), Human Metapneumovirus, Adenovirus, Coronavirus, Bocavirus and *Enterovirus* genus (HRV and HEV) using a multiplex nucleic acid amplification panel (xTAG RVP FAST v2, (Luminex Molecular Diagnostics, Austin, TX, USA)). Rapid direct RSV antigen detection was also performed using Alere BinaxNOW<sup>®</sup> RSV Card (Abbott Diagnostics).

Among the collected samples, 104 were positive for HRV and/or HEV using the multiplex test. Aliquots of these samples were transported to the laboratory of Microbiology of BHC Hospital for HRV and HEV molecular distinction and identification.

### *HRV and HEV real-time PCR detection*

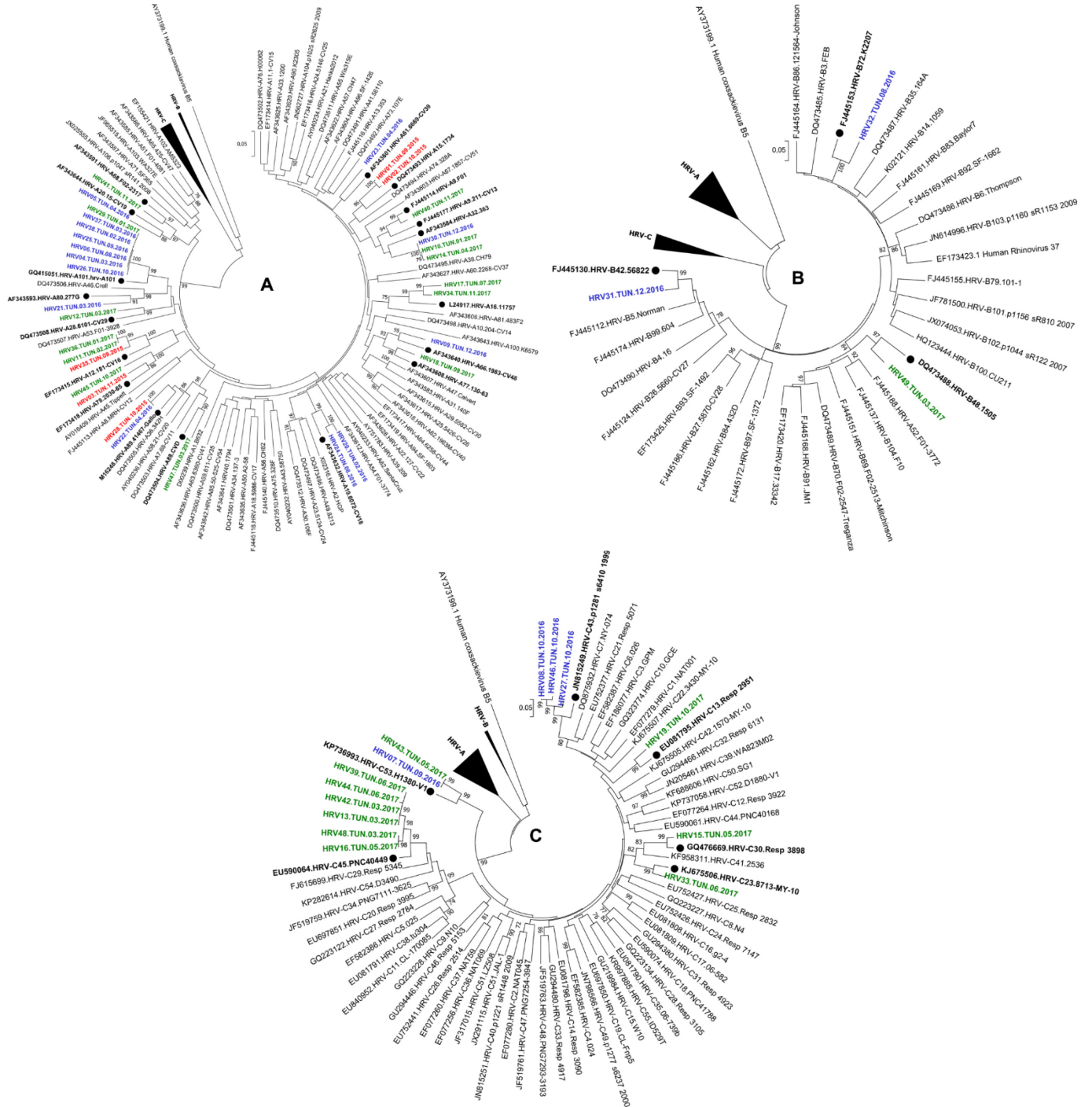
Viral RNA was extracted using the QIAamp MinElute Virus Spin Kit (Qiagen, GmbH, Hilden, Germany) according to manufacturer's recommendations.

Distinction and specific detection of HRV and HEV was assessed by two in-house real-time PCRs (RT-PCR) realized in two separate tubes. Primers and probes of HEV and HRV were selected, among those previously published, after validation by bioinformatic tools: Blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Primer Express software version 3.0.1 (Applied Biosystems, Life technology, USA), in order to assess their nucleotide specificity and to evaluate specific criteria for the design of primer and probe to be used in RT-PCR. For HEV RT-PCR, the selected primers and probe were EV1 (5'CCTGAATGCGGCTAATCC-3'), EV2 (ATTGTCACCATAAGCAGCC) and EVHP probe (HEX-ACCGACTACTTTGGGTGTCCGTGTTTC-3'-BHQ1) designed by Volle *et al.* [20]. For HRV

detection, we selected Rhv1-F1 (5'-CPXGCCZGCGTGGC-3'), Rhv1-R1(5'-GAAACACGGACACCCAAAGTA-5') and Rhv1-P (FAM-5'-TCCTCCGGCCCCTGAATGYGGC-3'-

BHQ1) previously published by Lu *et al.* [21]. The QuantiTect™ Probe RT-PCR Kit from Qiagen (GmbH, Hilden, Germany) was used for amplification. Briefly and based on a 25µL reaction volume, the PCR mixture

**Figure 1.** Molecular typing of HRV strains detected in investigated SARI cases.



**A.** Molecular typing of HRV-A strains; **B.** Molecular typing of HRV-B strains; **C.** Molecular typing of HRV-C strains. The evolutionary history was inferred using the Neighbor joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA 6. Sequences indicated in red, blue and green are Tunisian investigated sequences obtained in 2015, 2016 and 2017 respectively; sequences indicated in bold and with a black circle are the most related reference strains sequences.

contains 2µL of extracted RNA sample, 12.5µl of 2X Master Mix, 0.5µM of each primer, 0.1µM of probe, 0.25µL of Quanti Tect RT Mix, and RNase-free water if necessary. The reaction was performed in Lightcycler 480 instrument (Roche Diagnostic) during a reverse transcription step at 50°C (20 minutes), an initial incubation step at 95°C (15 minutes) and 40 cycles of a denaturation step at 94°C (15 seconds) and a combined annealing and extension step at 60°C (1 minutes).

Amplification data was analyzed by instrument software (Lightcycler 480II, Roche Diagnostic). A sample was considered as PCR-positive when its DNA amplification occurs at a crossing point value less than or equal to 35. If the crossing point value was between 35 and 40, it was considered as inconclusive result and was retested once again.

*HRV PCR amplification and sequencing*

Type identification was based on VP4/VP2 junction sequence analysis [15, 16]. This region was amplified using the primer pair 9565-reverse and 9895-forward, producing a fragment of approximately 549 nucleotides [15]. The PCR reaction was performed using the One Step RT-PCR kit from QIAGEN (GmbH, Hilden, Germany) in a total mixture of 25µl containing 2.5 µL of extracted RNA sample, 5µl of RT-PCR buffer, 400µM of each dNTP, 1µM of each primer, 1 µL of enzyme Mix, and RNase-free water if necessary. The thermal cycling conditions were as follows: 20 minutes at 50°C for reverse transcription, 15 minutes at 95°C for initial denaturation, 35 cycles of 1 minute at 95°C for

denaturation, 1 minute at 60°C for annealing, 1 minute at 72°C for extension, and 7 minutes at 72°C for a final extension step.

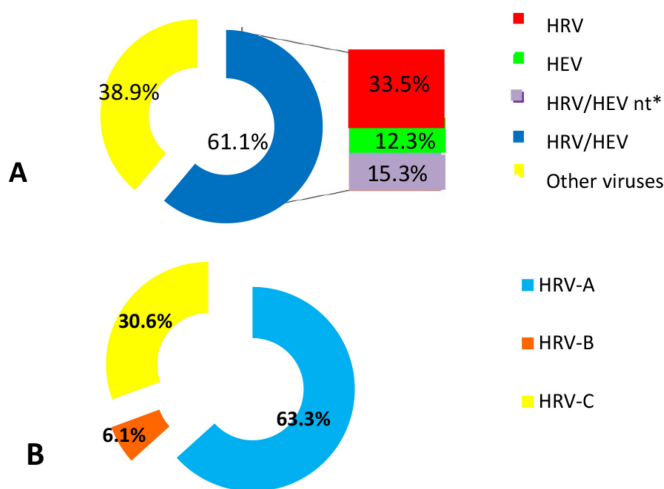
The VP4/VP2 amplicons were first visualized in ethidium-bromide containing 1% agarose gels. The amplicons of expected size (approximately 549 bp) were purified by the Qiaquick PCR purification kit from Qiagen (GmbH, Hilden, Germany). Sequences were derived by automated sequencing with Big Dye terminator chemistry according to the manufacturer’s protocol (Applied Biosystems) and reaction products were analyzed in an automated sequencer (ABI 3130).

*Sequence Analysis and type attribution*

Sequence data was analyzed using Clustal X program (<http://www.clustal.org/>) for multiple alignments and Mega version 6.0 (<https://www.megasoftware.net/>) for phylogenetic analysis. Dendrograms were drawn using the Neighbor-joining method and confirmed with 1000 bootstrap replicates. The sequences reported in this study were submitted to GenBank database: accession numbers MN583126 to MN583174 were assigned to sequences of HRV1 to HRV49 respectively.

HRV type identification was established using the VP4/VP2 partial regions [16] in comparison with sequences of reference strains of HRV-A, B and C available at the Online 10<sup>th</sup> report of the International Committee on Taxonomy of Viruses [4]. Their accession numbers were indicated into dendrograms in Figure 1. It is confirmed when the considered strain shows less than 10% nucleotide divergence with the closest sequence of reference strain [14-16].

**Figure 2.** General rates of HRV SARI cases.



**A.** Detection rates of HRV; **B.** Rates of HRV genogroups. nt: not typed (no residual sample available for typing); Other virus: specimens positive for Respiratory Syncytial Virus, influenzae A and B virus, Parainfluenzae virus, Bocavirus, Adenovirus, Metapneumovirus.

*Statistical Analyses*

The representation of patient’s data and detected pathogens were analyzed using the Epi Info 7.2 statistical package (<https://www.cdc.gov/epiinfo/support/downloads.html>). The relation and differences between pathogens infection rates, clinical manifestations and the statistical calculations were performed using the Chi-square ( $\chi^2$ ) test or the Fisher’s exact test (where cell counts below 5 were encountered in the statistical table). A value of  $p \leq 0.05$  was considered as significant.

**Results**

*Virus detection rate and Epidemiological features*

The age of the 271 children enrolled in this study ranged from 1 day to 144 months with a median age of 2 months. The male to female ratio was 1.27.

Among these specimens, at least one respiratory virus was detected in 170 cases (62.7% of SARI cases). One hundred and four samples were positive for both HRV and HEV by the multiplex nucleic acid amplification panel, corresponding to 61.1% of viral cases. Among HRV and/or HEV positive specimens, 57 cases (33.5%) and 21 (12.3%) were positive for HRV and HEV, respectively. For 26 cases, discrimination between HRV and HEV was not possible due to loss of residual samples for testing (Figure 2).

HRV and HEV positive samples were obtained from children aged from one week to 4 years primarily under 2 years of age (77.2%) with a further distinction of 64 boys and 40 girls. Regarding HRV positive samples, they were obtained from children aged

primarily under 6 months of age (77.2%) and especially in boys (34 boys and 23 girls) (Table 1).

#### HRV type identification

Among the 57 HRV positive samples, 49 had sufficient residual samples and were amplified and sequenced. The analysis of VP2-VP4 region revealed that HRV-A were the most frequent (63.3%) followed by HRV-C (30.6%) and only three cases of HRV-B (6.1%). Results showed circulation of twenty-seven different confirmed types from September 2015 to November 2017 (Figure 1). The most prevalent and diversified specie was HRV-A, represented by eighteen different types (HRV-A9, 12, 15, 16, 19, 20, 22, 28, 32, 61, 66, 68, 77, 78, 80, 88, 89, 101). HRV-A101 was the

**Table 1.** Demographic data and clinical characteristics of HRV SARI cases in northern Tunisian children.

	HRV-A (N = 31)	HRV-B (N = 3)	HRV-C (N = 15)	P	HRV (N = 57)	Viral infection (N = 170)
<b>Gender (M/F)</b>	1.06 (16/15)	(3/0)	1.5 (9/6)		1.4 (34/23)	1.32 (97/73)
<b>Age</b>						
Median (months)	2.15	7	2		2.4	2
< 6 months n (%)	25 (80.6)	1 (33.3)	14 (93.3)	<b>0.048</b>	44 (77.2)	134 (78.8)
6-12 months n (%)	2 (6.5)	1 (33.3)	1 (6.7)	0.9	5 (8.8)	13 (7.6)
12-48 months n (%)	1 (3.2)	0	0	0.06	3 (5.3)	14 (8.2)
missing	3 (9.7)	1 (33.3)	0	-	5 (8.8)	9 (5.3)
<b>Clinical signs n (%)</b>						
Fever	10 (32.3)	0	6 (40)	0.41	18 (31.6)	52 (30.6)
Cough	8 (25.8)	1 (33.3)	3 (20)	0.9	13 (22.8)	64 (37.6)
Dyspnea	21 (67.7)	1 (33.3)	7 (46.7)	0.21	32 (56.1)	114 (67.1)
Vomiting	0	1 (33.3)	0	0.67	5 (8.7)	8 (4.7)
Diarrhea	1 (3.2)	0 (33.3)	1 (6.7)	0.08	4 (7)	8 (4.7)
Apnea	3 (9.7)	0	1 (6.7)	0.85	4 (7)	16 (9.4)
Cyanosis	4 (12.9)	0	2 (13.3)	0.75	7 (12.3)	21 (12.4)
<b>Underlying risk factors n (%)</b>						
Chronic respiratory disease	0	0	0	-	0	1 (0.6)
Recurrent respiratory infection	0	0	0	-	0	2 (1.2)
Prematurity (< 36 weeks)	8 (25.8)	0	2 (13.3)	0.42	13 (22.8)	29 (17.1)
Immunocompromised	0	0	0	-	0	2 (1.2)
Chronic heart diseases	5 (16.1)	1 (33.3)	4 (26.7)	0.40	10 (17.5)	18 (10.6)
Metabolic disorder	1 (3.2)	0	0	0.74	1 (1.8)	2 (1.2)
<b>Viral coinfection n (%)</b>	3 (9.7)	1 (33.3)	2 (13.3)	0.48	6* (10.5)	12 (7.1)
<b>Bacterial coinfection n (%)</b>	5 (16.1)	2 (66.6)	4 (26.7)	0.14	11 (19.3)	37 (21.8)
<b>Seasonal distribution n (%)</b>						
Spring-Summer	10 (32.2)	2 (66.7)	9 (60)	0.31	25 (43.9)	59 (34.7)
Autumn- Winter	21 (67.8)	1 (33.3)	6 (40)	<b>0.05</b>	32 (56.1)	111 (65.3)
<b>Death n (%)</b>	1 (3.2)	0	1 (6.7)	0.71	2 (3.5)	8 (4.7)
<b>Non invasive positive pressure ventilation</b>	7 (22.6)	1 (33.3)	1 (6.7)	0.39	10 (17.5)	40 (23.5)
<b>Intubation</b>	9 (29)	1 (33.3)	8 (53.3)	<b>0.028</b>	18 (31.6)	66 (38.8)
<b>Duration of hospitalization in PICU</b>						
< 4 days	8 (25.8)	0	1 (6.7)	-	12 (21.1)	33 (19.4)
4-7 days	2 (6.5)	0	2 (13.3)	-	4 (7)	27 (20.6)
> 7 days	10 (32.3)	2 (66.7)	6 (40)	0.36	19 (33.3)	71 (41.8)

HRV: Human Rhinovirus; HEV: Human Enterovirus; p: statistically significant difference between HRV-A; HRV-B and HRV-C; \*One case with both viral and bacterial co-infection.

most prevalent type (7/32), circulating in Tunisia during at least two consecutive years followed by other types (HRV-A12, HRV-A78, HRV-A32 and A89). Whereas, other types seem to be more sporadic and were detected only one time (HRV-A61; A9; A66; A77; A88; A28; A80; A68; A20) or during a limited period (HRV-A15, A16, A19). HRV-B types were rarely detected in the study: only three types were obtained (HRV-B72, B48 and B42). Regarding HRV-C, six different types were detected during the study period: HRV-C43, C13, C30, C23, C45 and C53. HRV-C45 was the most circulating strains (6/14) in contrast with HRV-C13, C30 and C23 were only detected once.

*Age and gender distributions of HRV infection*

The majority of HRV cases were found in toddlers less than 6 months (71.4%). The incidence was statistically significant for HRV-A detection before the age of six months where 80.6% of cases were positive ( $p = 0.048$ ). No significant difference by gender distribution of HRV and respective types were observed (Table 1).

*Clinical features and risk factors*

The comparison of symptoms and underlying risk factors with HRV infection and patients without infection showed no significant difference. Nevertheless, HRV-C infection was associated with complicated infection requiring tracheal intubation ( $p = 0.028$ ) (Table 1).

*Bacterial and viral Co-infection*

From the 57 HRV positive cases, a total of 16 cases (28%) were found with co-infection (bacterial and/or viral). Five cases (8.7%) were found in co-infection with one other respiratory virus: RVS (2/57), Bocavirus (2/57) and Parainfluenzae virus (1/57) and 10 cases (17.5%) in co-infection with one bacterium among: *Streptococcus pneumoniae* (4/57), *Haemophilus influenzae* (4/57), *Moraxella catarrhalis* (1/57) and *Bordetella pertussis* (1/57). In addition, one case was found with both viral and bacterial coinfection (Parainfluenza virus and *Streptococcus pneumoniae*). Complications including intubation and a prolonged stay in intensive care (> 7 days) were significantly associated with bacterial co-infection in HRV-A cases ( $p = 0.05$  and  $p = 0.03$  respectively).

*Seasonal HRV types distribution*

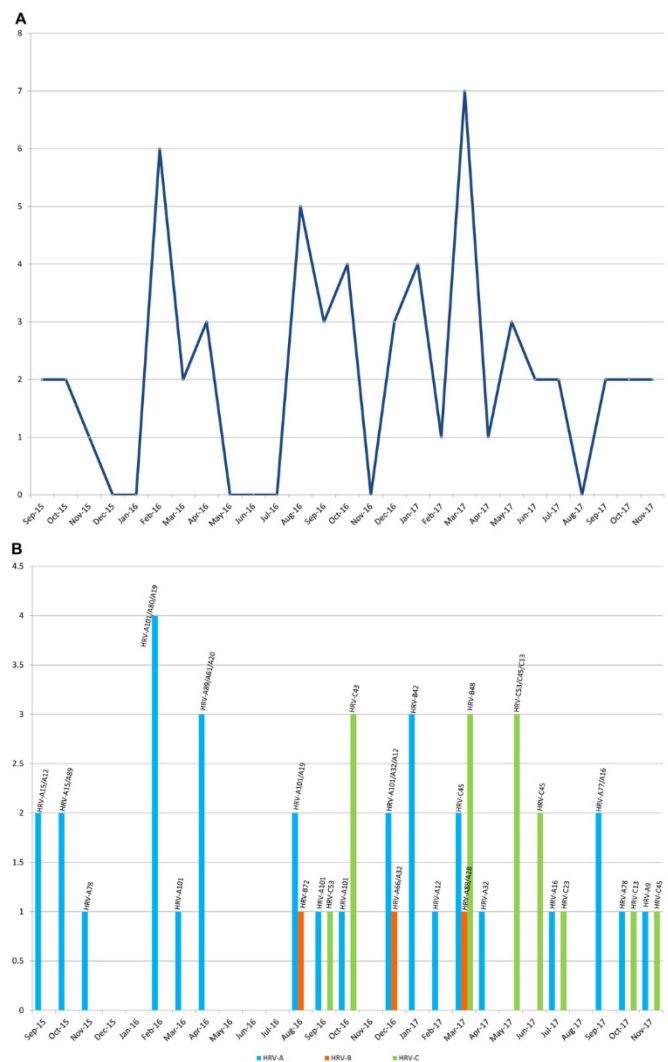
Figure 3 shows the temporal distribution of HRV between September 2015 and November 2017. HRV was common during the autumn, winter and spring.

Regarding HRV-A types, they were detected throughout the three years of the study, particularly in autumn and winter ( $p = 0.05$ ) (Figure 3B, Table 1). In contrast, HRV-B types were sporadically detected in August and December 2016 and March 2017. HRV-C types were only detected during a limited one-year period (September 2016- November 2017).

**Discussion**

HRV is among the leading causes of SARI in infants, however, its burden and genotypic diversity vary from one region to another and little is known in northern African regions. In view of the paucity of available information, this study describes epidemiological, clinical patterns and genotypic

**Figure 3.** Temporal distribution of HRV strains detected between September 2015 and November 2017.



**A.** Temporal distribution of all HRV species; **B.** Temporal distribution of different HRV types.

diversity of HRV in SARI cases in northern Tunisian regions. It contributes, for the first time, to a better understanding of the HRV circulation especially in North-Africa.

Our study shows that respiratory picornaviruses constituted the main cause of viral SARI cases (61%) in northern Tunisian children between September 2015 and November 2017. This result is in line with previous investigations of respiratory infection in Tunisian population, where HRV and/or HEV infection constituted 43% and 61% in 2009-2010 and 2010-2011 respectively [22]. However, HRV rate totaling approximately a third of cases is different from previously recorded results. Indeed, between September 2013 and December 2014 in the central Tunisian region, HRV was considered as the leading cause of SARI reaching more than 50% of the investigated cases [17] and also in another North African country eg. Morocco (November 2010-December 2011) [23]. At the same time, present results are similar to results obtained from January 2009 to March 2010 in the south of Tunisia [18] and in others studies targeting subtropical and western regions of China [24,25] or also in Jordan [26]. These differences in detection rates may be explained by: i) the specificity of the used assay which may lead to false positive detection; ii) the epidemiological and demographic variabilities from a region to another. Indeed, demographic patterns, access to healthcare and connectivity may lead to differences in detection rates. It's also assumed that different bioclimatic and environmental factors may affect virus incidence.

The distribution of HRV cases per age group showed a decrease of infection rates according to the age. This might be explained by cumulative immunity in result of successive exposition to different serotypes. In the other hand, most cases occurred before six-months of age, particularly for HRV-A infection. Other studies corroborate the same finding such as in western China where the highest HRV infection rate was before six months' age [24] or in South Africa where 60.9% of positive cases were isolated from the same age group [27]. Meanwhile, other studies demonstrated that HRV infection occurs later, between 13 and 36 months [25,28]. It's probable that younger children are more susceptible to infection than older children due to the immaturity of the immune system. Furthermore, infection in younger children could be, in general, complicated and requiring hospitalization while in older children, HRV infections are likely to be benign and consequently not included in such studies. Otherwise, HRV infections in younger children may also be

enhanced by close contact with infected adults which is a common practice in our region. Additionally, the general decrease of breastfeeding among working women in cities similarly to the investigated region in this study, may also impact the incidence of HRV infection in young children. Thus, our finding highlights the high infection rate in the first year of age and the need to exercise more care and the implementation of appropriate preventative measures at this age.

In our study, complicated infections requiring intubation and long stays in intensive care unit were associated to HRV-C and HRV-A with bacterial co-infection. Previous studies suggested involvement of HRV-C in more severe illness such as pneumonia, asthma and exacerbation of asthma [29]. Indeed, recent studies show that HRV can induce injuries by acting at different stages: overexpression of a range of cytokines (IL-1 $\beta$ , 6, 11), growth factors (G-CSF, GM-CSF), and chemokines (CXCL8, 5, 10) which can enhance airway inflammation and the potential to accelerate preexisting allergic inflammation [30]. Nevertheless, corroboration of the hypothesis for the greater pathogenicity of species C will require larger number of subjects using control groups. Regarding bacterial co-infection, it can reach 70% and is frequent in younger children given their developing immune system [31]. It could be caused by simultaneous infection from different microbes or by concomitant new infection with a pre-existent asymptomatic bacterial or viral carriage [32]. Other reports indicated that co-infection may lead to more severe infection compared to a single infection, especially in the case of RSV co-infection [17] or bacterial co-infection [33]. It can be inferred that viral infection could facilitate bacterial invasion and pathogenesis through many mechanisms, such as disruption of the epithelial barrier, over expression of adhesion proteins and general alteration of the immunity leading to longer hospital stays [34]. In this study, bacterial co-infection cases due to *Streptococcus pneumoniae* and *Haemophilus influenzae* were the most prevalent and complicated. Noticeably, this study was achieved prior to the introduction of pneumococcal vaccine in April 2019 in Tunisia [35]. This vaccine is believed to substantially reduce the burden of pneumococcal disease. Thus, it may be relevant to evaluate the impact of such vaccination on SARI, in the future.

The three HRV groups were detected among hospitalized northern Tunisian children with predominance of HRV-A species (63.3%) followed by HRV-C (30.6%) and HRV-B (6.1%). Similar results

were generally obtained among hospitalized children in rural Thailand [36], Australia [37] and South Africa [38]. However, among adults, the distribution of HRV groups seems to be different with higher prevalence of HRV-B. For instance, in Thailand, for age groups more than 20 years, 19 to 39% of HRV-B and 13 to 33% of HRV-C were detected [36]. High diversity was observed among circulating HRV strains in northern Tunisia as demonstrated in other regions of the world eg. Africa [38], Europe, America and also Asia [39,40]. This diversity may result from the high genetic evolution level of picornaviruses, in general. Furthermore, the mild clinical manifestations of HRV, in adults, may lead to continuous activity and circulation of diseased persons, allowing the spread of different strains concurrently.

This study demonstrated, for the first time, the circulation of highly prevalent types in the world (HRV-A12, A78 and A101 and HRV-C43 and C45), also, in Tunisia and, consequently, in North African region. Interestingly, it revealed the circulation of rarely detected types in the world such as HRV-A77, A88 and HRV-C13, C30, C53 [14]. The majority of HRV types were detected during a limited time period. Thus, they may spread after a recent importation event. However, other types (HRV-A101, A12, A78, A32, A89 et HRV-C45) circulated during an extended period with continuous circulation or turnover of these types. This pattern may be due to: i) the endemic presence of these virus types in the region; ii) importation followed by the spread of the virus type in the region; iii) or, also, multiple importation events of different virus lineages of the same type during the studied period. We cannot also exclude the possibility of the presence of endemic circulation of these strains and in the same time importation from another country of virus from the same type. Further phylogenetic and phylogeographic studies may help to evaluate diversity and trace their epidemiological origin.

This study allowed description of epidemiological and clinical patterns of HRV in SARI cases and the diversity of types circulating in northern Tunisian regions. Nevertheless, it included some limitations. Indeed, it was difficult to assess identification of all HRV cases, given the loss of residual samples for testing. The collected samples were first divided into two aliquots for an initial routine diagnostic: an aliquot for bacterial diagnostic and another for virologic diagnostic. Then, an aliquot for our research was prepared. In the future, it will be interesting to focus on respiratory HEV isolation and typing which could reveal HEV types associated with respiratory infections

and improve our general knowledge on circulating HEV in Tunisia.

## Conclusions

During the study period, respiratory picornaviruses (HRV and HEV) constituted a major cause of SARI in Tunisian children concentrated in children under one year of age. This finding highlights the need for appropriate preventative measures during the first year of life. To the best of our knowledge, this is the first study describing the high diversity of HRV types in northern Tunisia, a North African region, providing more knowledge on HRV infections in those regions. Nevertheless, deeply phylogenetic studies of different detected types may help to precisely identify the diversity of different types and trace their epidemiological origin.

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