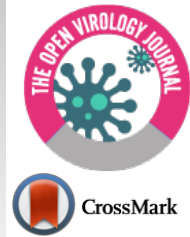




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## LETTER

### Impact of Enterovirus Molecular Assay Turnaround Time on Hospitalization Length During an Echovirus 30 Meningitis Outbreak, France, Fall 2014

Yohan N'Guyen<sup>1,2,\*</sup>, Anne L. Lebreil<sup>1</sup>, Philippine Simphal<sup>2</sup>, Christine Pietrement<sup>3</sup>, Nathalie Bednarek<sup>4</sup>, Pauline Orquevaux<sup>2</sup>, Paul A. Gretteau<sup>1</sup> and Laurent Andreoletti<sup>1</sup>

<sup>1</sup>Laboratoire de Virologie EA 4684, UFR Médecine, Université Reims Champagne Ardennes, 51 rue Cognacq Jay 51100 Reims, France

<sup>2</sup>Service de Médecine interne, maladies infectieuses et Immunologie Clinique, Hôpital Robert Debré, Avenue Général Koenig 51100 Reims, France

<sup>3</sup>Service de Pédiatrie Générale et spécialisée, American Memorial Hospital, 47 Rue Cognacq-Jay, 51100 Reims, France

<sup>4</sup>Service de Médecine Néonatale et Réanimation Pédiatrique, Institut Mère Enfant Alix de Champagne, 45 Rue Cognacq-Jay, 51100 Reims, France

#### Abstract:

#### Background:

The impact of Enterovirus Real Time-Polymerase Chain Reaction assay (EV RT-PCR) on hospitalization lengths of patients with aseptic meningitis has been investigated but the impact of early EV RT-PCR results released on time before patient discharge remains unclear during Echovirus meningitis outbreaks.

#### Objective:

To assess a potential correlation between EV RT-PCR turn-around time and hospitalization lengths during an Echovirus meningitis outbreak.

#### Method:

Eighteen patients demonstrating a positive EV RT-PCR assay performed on Cerebrospinal Fluid (CSF) samples collected between October 1<sup>st</sup> 2014 and December 31<sup>st</sup> 2014 were retrospectively included. Viral protein 1 (VP1) gene region was amplified and sequenced using a classical Sanger sequencing reaction. Clinical data were retrospectively collected from patient's records. Quantitative variables expressed as median values and ranges were compared using Mann Whitney U test. Correlations were performed using simple regression analysis.

#### Results:

Phylogenetic VP1 sequence analyses identified that the outbreak was related to an Echovirus 30 strain in 7 out of the 10 cases with available sequencing data. The three remaining sequences analyses evidenced Echovirus 14, 9 and 7 strains. Hospitalization length was statistically shorter in children without comorbidity (n=5) than in adult patients (n=10) or neonates and children with comorbidity (n=3) (p=0.003 and 0.01 respectively), whereas EV RT-PCR turnaround time was not statistically different between these groups. Correlation between hospitalization length and EV RT-PCR turnaround time was poor (R<sup>2</sup>=0.06), especially in adults (R<sup>2</sup>=0.01)

#### Conclusion:

Our data indicated that EV RT-PCR turnaround time was not correlated to hospitalization length during a short Echovirus meningitis outbreak.

**Keywords:** Hospitalization length, Enterovirus, Outbreak, Aseptic meningitis, Real-time polymerase chain reaction, Cerebrospinal fluid.

#### Article History

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## 1. INTRODUCTION

Echoviruses (species Enterovirus B, genus *Enterovirus*, family *Picornaviridae*) are the most common cause of aseptic

meningitis in children and adult patients usually occurring by epidemics during summer and autumn months in Western countries [1 - 5]. Even though there is no available specific antiviral treatment that could have a significant clinical impact on patients [6], overall prognosis of meningitis due to echoviruses is good [3].

However, aseptic meningitis cases due to echovirus are

\* Address correspondence to these authors at the Avenue Général Koenig, 51100 Reims, France; Tel: (+33)326789422; Fax: (+33)326784090; Email: yohan.nguyen@wanadoo.fr

frequently hospitalized for a few days, the time required to rule out bacterial meningitis and to confirm enteroviral meningitis. Such hospitalization is expensive and numerous past studies have studied the impact of Enterovirus (EV) Real Time-Polymerase Chain Reaction (RT-PCR) assays that have definitively supplanted viral culture methods [7] on hospitalization length. In house, daily testing protocols as well as fully automated assays allowing point of care testing have been associated with shorter hospitalization length than send out, three times a week and conventional PCRs assays with manual extraction step [8 - 10]. Retrospective analysis of medical records also suggested that echovirus meningitis cases with EV RT-PCR results released within 24 hours tend to have a shorter hospitalization length than cases with EV RT-PCR results available in more than 24 hours [7, 11], but the benefit of early RT-PCR reports is not so obvious during EV meningitis outbreaks [4, 12]. Indeed during outbreaks, patients are often discharged before RT-PCR results in case of apparently negative bacterial culture of the cerebrospinal fluid [12]. Conversely, the occurrence of postlumbar puncture intracranial hypotension could be associated with prolonged hospitalization length [13] despite early RT-PCR results.

Herein, we reported data from an unusual welldefined outbreak of aseptic meningitis related to echovirus 30 in a single university hospital. In the present study, we investigated specifically (i) the presence of correlation between EV RT-PCR assay turnaround time and hospitalization length (ii) the occurrence of postlumbar puncture intracranial hypotension (iii) hospital discharge rates before RT-PCR results. Our present investigation aimed to assess the consequences of early EV RT-PCR reports onto the time delay before patient discharge during short or unusual EV outbreaks.

## 2. MATERIAL AND METHODS

### 2.1. Context

In France, in 2014, the number of cases of Enterovirus infections (mainly aseptic meningitis) during autumn was unusually high compared to those of previous or following years (Fig. 1A). In Reims University Hospital, the number of cases of aseptic meningitis with positive EV RT-PCR assay during the last trimester 2014 was also higher than those observed in the last trimester 2013 (see the results section). During the last trimester 2014, diagnostic and therapeutic strategies in Reims University Hospital remained unchanged: in house EV RT-PCR assay was performed three times a week (Monday, Wednesday, Friday) in Virology Laboratory using the commercially available Enterovirus R-gene kit (Biomérieux®, Varhiles, France) and treatment prescription remained at patient's physician discretion.

### 2.2. Inclusion Criteria

All patients with positive EV RT-PCR assay performed on Cerebrospinal Fluid (CSF) samples collected by lumbar puncture between October 1<sup>st</sup> 2014 and December 31<sup>st</sup> 2014 were included. Subsequent CSF samples collection for already included patients was not taken into account.

### 2.3. Data Collection and Definition

Clinical data were retrospectively collected from patient's records by an external reviewer (PS). Comorbidity was defined as the presence of chronic disease or background with potential impact on overall health status (see the results section).

### 2.4. Ethics

No objection was raised by any of the study patients in accordance with French Legislation (Loi Jarde). The Hospital Ethics Committee approved this retrospective study and investigations conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

### 2.5. Sequencing Analysis

CSF samples of included patients were extracted using QIA amp® Viral RNA Mini-Kit. Viral protein 1 (VP1) and 3D Polymerase (3D-Pol) gene regions were then amplified, as described by Nix *et al.* [14] and Lukashchuk *et al.* [15] respectively. Amplicons were purified using Purelink Quickgel extraction kit according to manufacturer's instructions (ThermoFischer scientific®, Illkirch, France) before a Sanger sequencing reaction was performed [16] (Genewiz®, Takeley Essex, United Kingdom). All available human echovirus E30 VP1 (n=13) and 3DPol (n=15) partial genomes were downloaded from Genbank (<https://www.ncbi.nlm.nih.gov/genbank/>) on 2/25/18. Sequences were analyzed as described by McIntyre and colleagues [17], 2013. Briefly, regions with more than 90% coverage were analyzed between positions 2581- 2873 for VP1 and 5887-6461 for 3DPol (all numbering are reported following the reference strain Echovirus 30 strain Bastianni (accession number KY888275.1)). Sequence alignments were performed with Mega version 7.0 (Molecular Evolutionary Genetics Analysis) [18], using ClustalW algorithm and manual inspection of nucleotide alignments. Phylogenetic analysis was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [19] and a bootstrap method of 1,000 iterations. Distribution of pairwise nucleotide p-distances was then calculated for individual regions. Echovirus 30 VP1 sequences are available under the GENBANK numbers: MH 307969- MH307975; Echovirus 30 3Dpol sequences are available under the Genbank numbers MH307962-MH307968.

### 2.6. Statistical Analyses

Qualitative variables expressed as percentages were compared using Fischer's exact test or Chi<sup>2</sup> Pearson's test if applicable. Quantitative variables expressed as median values and ranges were compared using the Mann Whitney U test. Correlations were performed using simple regression analysis. Statistical analyses were performed using Statview 5.0 software (SAS Institute). A p-value <0.05 was considered significant.

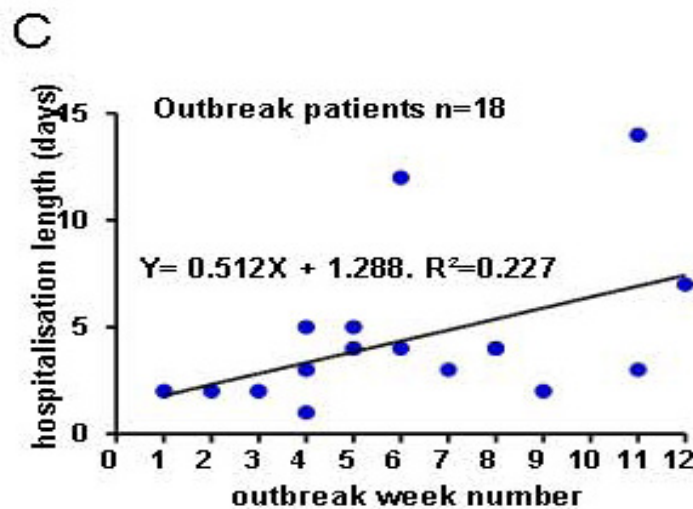
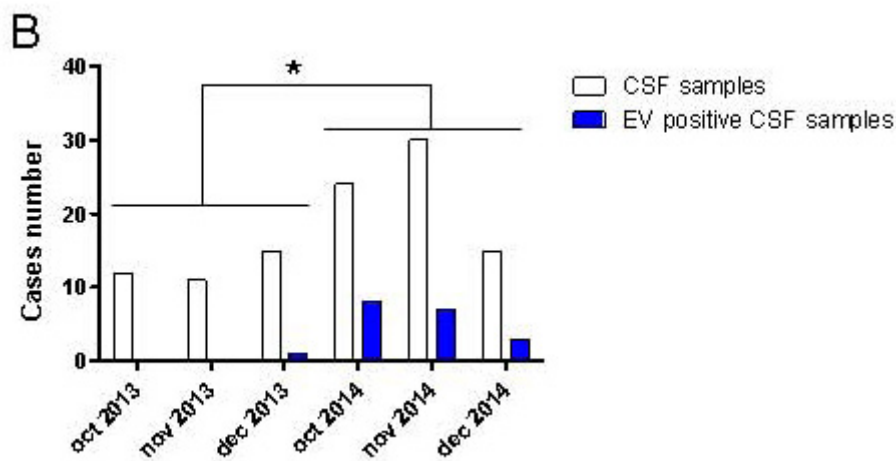
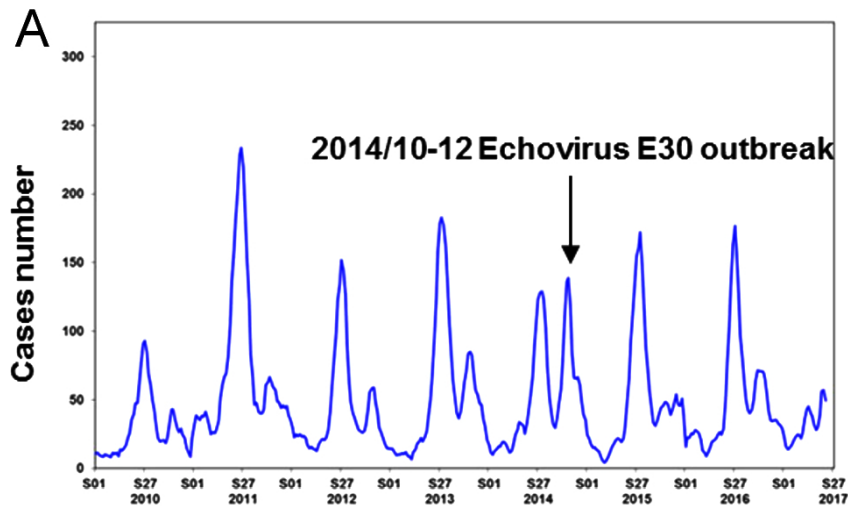
## 3. RESULT

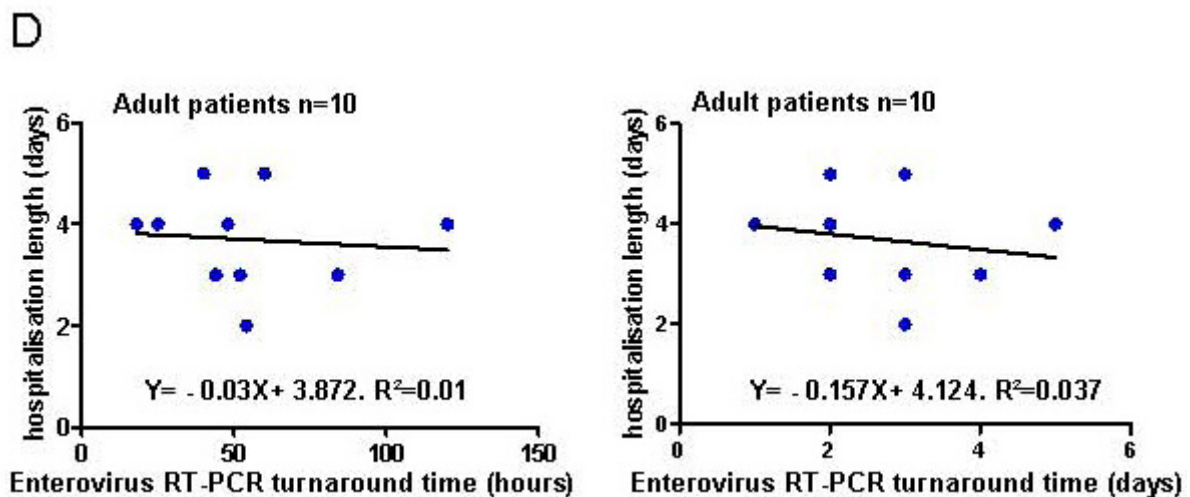
### 3.1. Description of the Outbreak Occurring in Reims University Hospital During the Last Trimester of 2014

Eighteen out of 69 patients (26.1%) who had a lumbar

puncture between October 1<sup>st</sup> 2014 and December 31st 2014 were “enterovirus positive” whereas only one of the 38 patients (2.6%) who had lumbar puncture between October 1<sup>st</sup> 2013 and December 31st 2013 was “enterovirus positive” (p=0.0024, according to Chi<sup>2</sup> Pearson’s test) (Fig. 1B).

During the last trimester of 2014, 8 cases occurred in October, 7 in November and 3 in December. Thirteen out of 18 outbreak patients were living in Reims urban district. Sex ratio was 1.25 (10 males and 8 females), median age was 17 years [4 days 63 years]. Eight cases documented in children of less than 15 years-old were managed in the department of pediatrics.





**Fig. (1).** **A**) Number of cases per week of Enterovirus infections (mainly aseptic meningitis) according to Enterovirus Survey Network in France from January 1<sup>st</sup> 2010 to June 10<sup>th</sup> 2017 (data issued from <http://invs.santepubliquefrance.fr/Dossiers-thematiques/Maladies-infectieuses/Maladies-a-prevention-vaccinale/Poliomyelite/Points-de-situation/Point-sur-les-infections-a-enterovirus-au-20-juin-2017>, accessed on February 19<sup>th</sup> 2018.) Black arrow depicted number of cases corresponding to autumn 2014. **B**) Number of cases per month of Cerebrospinal Fluids (CSF) sampled and Enterovirus (EV) positive Cerebrospinal Fluids sampled in Reims University Hospital during the months of October (oct), November (nov) and December (dec) 2013 and 2014. \*  $p < 0.05$ . **C**) Correlation between hospitalization length (days) and outbreak length expressed as the number of weeks outbreak was pursuing. Outbreak week number 1 to 4 corresponded to the month of October 2014, 5 to 8 to the month of November 2014 and 9 to 12 to the month of December 2014. **D**) Correlation between hospitalization length (days) and Enterovirus real time polymerase chain reaction (EV RT-PCR) turnaround time in adult patients ( $n=10$ ). Left, EV RT-PCR turnaround time expressed in hours, right, EV RT-PCR turnaround time expressed in calendar days.

### 3.2. Sequencing Analysis

Although all of the 18 patient's samples were tested, sequencing analysis data were available for 10 out of the 18 (55.6%) outbreak patients. Phylogenetic VP1 sequences analyses identified that the outbreak was related to an Echovirus 30 strain in 7 out of the cases (70%). The three remaining sequences analyses evidenced Echovirus 14, 9 and 7 strains. Patients with sequencing analysis data available were not clinically different from those without sequencing analysis data available (not shown). The phylogenetic tree constructed using the VP1 sequences is depicted in Fig. (2A). All of these strains belonged to the same cluster that was closer to strains circulating in France during 2008 and 2009 (nucleotide p-distance 0.024 to 0.046 and 0.038 to 0.065 respectively) than to strains circulating in France during 2011 or 2013 (nucleotide p-distance 0.041 to 0.072 and 0.045 to 0.078 respectively), strains circulating in Italy during 2014 (nucleotide p-distance 0.116 to 0.137) or Bastianni reference strain (nucleotide p-distance 0.202 to 0.209). Supplementary analysis of 3D-Pol viral gene region was performed in these 7 strains confirming the identification of an Echovirus 30 subtype and moreover suggesting the absence of large intra or inter-serotypic recombination events that could explain the occurrence of this autumnal outbreak (Fig. 2B).

### 3.3. Clinicobiological Data and Outcome

Cephalalgia was reported among all patients able to express it but fever was absent in 5 out of the 18 outbreak patients (27.7%). Neck stiffness was absent in 9 cases (50.0%)

whereas 3 patients had neurological symptoms (diplopia, seizure or nystagmus). Two newborns aged 4 and 20 days had systemic symptoms (sepsis, respiratory distress or myocarditis).

Comorbidity was evidenced in 5 out of the 18 patients (27.8%) (Asthma  $n=1$ , Chronic Obstructive Pulmonary Disease  $n=1$ , Obesity  $n=1$ , Leukodystrophy  $n=1$  and Pre-maturity (30 weeks gestation  $n=1$ ). Blood C reactive protein was greater than 10 mg/L in 14 outbreak patients (77.8%) whereas procalcitonin was lower than 0.5  $\mu\text{g/L}$  among all patients. Two patients had normal CSF white cell counts (11.1%) and 16 had clear CSF pleiocytosis, but only 8 out of these 16 (50%) had obvious CSF lymphocytic predominance. All bacterial cultures of CSF remained sterile. Only 3 out of the 18 outbreak patients (16.6%) had CSF protein concentration superior to 1g/L and CSF interferon alpha level was superior to 2UI/mL in 8 out of the 18 outbreak patients (44.4%). Anti-infective treatment (Antibiotics ( $n=7$ ); Aciclovir ( $n=2$ )) was prescribed in 7 cases (38.8%) and no acute renal failure or death occurred in any of the outbreak patients.

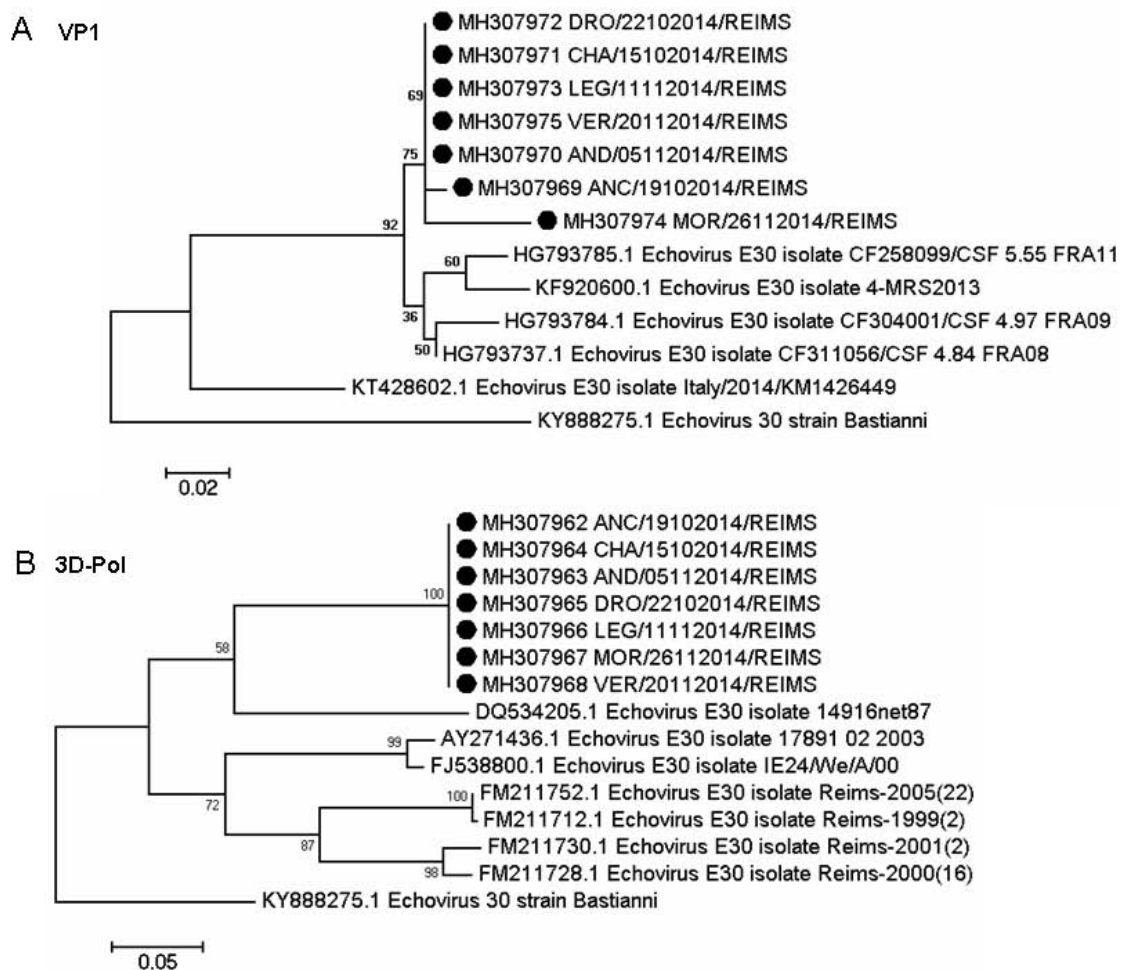
Clinicobiological data and outcome are shown in Table 1 for all outbreak patients that were categorized for descriptive purposes in: neonates and children with comorbidity ( $n=3$ ), children without comorbidity ( $n=5$ ) and adult patients ( $n=10$ ). Interestingly, hospitalization length was statistically shorter in children without comorbidity than in adult patients or neonates and children with comorbidity ( $p=0.003$  and 0.01 respectively), whereas EV RT-PCR turnaround time was not statistically different between children, adult and neonates.

This statistically significant difference between hospitalization lengths was related to higher rates of discharge from hospital before EV RT-PCR results among children without comorbidity than among adults or neonates ( $p=0.02$  and  $0.01$  respectively).

The correlation between hospitalization length and outbreak length is depicted in Fig. (1C). There was no clear trend to reduction of case's hospitalization length as the outbreak continued. Conversely, hospitalization length was statistically shorter in October than in the two last months (2 days [1 - 5] versus 4 days [2 - 14],  $p=0.02$ ) because cases involving children without comorbidity were overrepresented in October compared to the two last months (5 out of 8 (62.5%) versus 0 out of 10 (0%),  $p=0.006$  according to Fischer's exact test).

The correlation between hospitalization length and EV RT-

PCR turnaround time was poor ( $Y= - 0.34 X + 5.973$ ;  $R^2=0.06$ ,  $Y= - 0.131X+ 9.09$ ;  $R^2=0.16$ ,  $Y= - 0.03X+ 3.872$ ;  $R^2=0.01$ ) among all outbreak patients ( $n=18$ ), all children ( $n=8$ ) and all adults ( $n=10$ ) respectively. Because children without comorbidity were more frequently discharged before EV RT-PCR results and because neonates and children with comorbidity remained in hospitalisation despite EV RT-PCR results released (Table 1), only the correlation between hospitalization length and EV RT-PCR turnaround time in adult patients ( $n=10$ ) is depicted in Fig. (1D). Even in the absence of postlumbar puncture intracranial hypotension in adult patients (Table 1), the correlation between hospitalization length and EV RT-PCR turnaround time was poor ( $R^2= 0.01$ ). The correlation was not better ( $R^2 =0.03$ ) when EV RT-PCR turnaround time was expressed in calendar days, considering that medical decision to discharge a patient from hospital was a sequential process during rounds and not a continuous process.



**Fig. (2).** Phylogenetic trees comparing strains isolated during this outbreak ( $n=7$  and identified as Echovirus 30 strains according to reference Viral protein 1 gene region sequencing analysis) with available sequences of Echovirus circulating in Europe. **A)** Viral protein 1 (VP1) gene region. Black dots: strains isolated during outbreak: MH 307969- MH307975. **B)** 3D Polymerase (3D-Pol) gene region. Black dots: strains isolated during outbreak. MH307962-MH307968.

**Table 1. Clinicobiological data and outcome of outbreak patients. CRP: C reactive protein, CSF: Cerebrospinal Fluid. EV RT-PCR: Enterovirus Real-Time Polymerase Chain Reaction. \* Fischer's exact test. § Antibiotics (n=7), Aciclovir (n=2). Bold: significant differences.**

	All Patients (n=18)	A) Neonates and Children < 15 Years with Comorbidity (n=3)	B) Children < 15 Years without Comorbidity (n=5)	C) Adult Patients >15 years (n=10)	p B vs C	p B vs A
Male sex (n,%)	10 (55.55)	2 (66.66)	2 (40.00)	6 (60.00)	0.60*	0.99*
Reims urban district (n,%)	13 (72.22)	1 (33.33)	4 (80.00)	8 (80.00)	0.99*	0.46*
Comorbidity (n,%)	5 (27.77)	2 (66.66)	0 (0)	3 (30.00)	0.50*	0.10*
Apyrexia (n,%)	5 (27.77)	1 (33.33)	2 (40.00)	2 (20.00)	0.56*	0.99*
Absence of neck stiffness (n,%)	9 (50.00)	2 (66.66)	3 (60.00)	4 (40.00)	0.60*	0.99*
Median CRP (mg/L) [range]	16 [0.6-108]	55 [10.4-108]	26.5 [16-40]	12.5 [0.6-58]	0.25	0.47
Median White Blood cells count Blood(G/L) [range]	10.8 [4.7-22.2]	16.7 [13 - 22]	10.1 [9.9-15.4]	10.6 [4.7-16.7]	0.99	0.07
Median White Blood cells count CSF(n/mm3) [range]	67.5 [0-870]	35 [35-90]	26 [1-198]	149 [0-870]	0.32	0.65
<b>Median Protein concentration (mg/L) [range]</b>	<b>424 [166-1240]</b>	<b>1108 (684-1240)</b>	<b>198 [166-520]</b>	<b>424 [264-1040]</b>	<b>0.02</b>	<b>0.02</b>
CSF interferon alpha level >2UI/ml (n,%)	8 (44.44)	1 (33.33)	1 (20.00)	6 (60.00)	0.55*	0.99*
Median EV RT-PCR turnaround time (hours) [range]	47 [7-120]	21 [7-46]	48 [30-51]	50 [18-120]	0.38	0.09
<b>Discharged before EV RT-PCR Results released (n,%)</b>	<b>8 (44.44)</b>	<b>0 (0)</b>	<b>5 (100.00)</b>	<b>3 (30.00)</b>	<b>0.02*</b>	<b>0.01*</b>
Brain imaging prescribed (n,%)	6 (33.33)	1 (33.33)	0 (0)	5 (50.00)	0.10*	0.37*
<b>Median Hospitalization length (days) [range]</b>	<b>3.5 [1-14]</b>	<b>12 [7-14]</b>	<b>2 [1, 2]</b>	<b>4 [2-5]</b>	<b>0.003</b>	<b>0.01</b>
<b>Anti infective treatment prescribed§ (n,%)</b>	<b>7 (38.80)</b>	<b>3 (100.00)</b>	<b>0 (0)</b>	4 (40.00)	0.23*	<b>0.01*</b>
Post-lumbar puncture intracranial hypotension (n,%)	1 (5.55)	0 (0)	1 (20.00)	0 (0)	0.33	0.99*

#### 4. DISCUSSION

In the present study, our findings indicated that hospitalization length was poorly correlated to EV-RT PCR turnaround time during an Echovirus 30 aseptic meningitis outbreak occurring between October and December 2014. Short EV-RT PCR turnaround times were not systematically associated with short hospitalization lengths and significant discrepancies have been evidenced between children and adult patients, as previously described by Archimbaud and colleagues [7] in Clermont Ferrand reference centre during a whole year (summer outbreak and apart). Conversely, we focused this study only on an outbreak occurring during all autumn and the beginning of winter. Indeed, even if circulation of Enterovirus has already been reported during winter [5], we presumed such an unusual outbreak could have an impact of patient management because it could give to physicians the feeling of an ongoing outbreak earlier than during summer months.

According to VP1 region sequencing analysis, this unusual outbreak was mainly related to Echovirus 30 that is the main strain involved in aseptic meningitis outbreaks in France [20, 21] and in Europe [1, 22]. Supplementary sequencing of the 3D Pol did not support the hypothesis of an emerging new recombinant viral form [16, 17] but did not exclude the presence of intra or intertypic mosaic recombinant forms.

According to VP1 region sequencing analysis, the 7 outbreak strains that were belonging here to the same cluster were closer to strains circulating in France during 2008 and 2009 than more recent strains circulating in France or Europe. Previous massive sequencing data evidenced that echovirus trans-transportation was a major epidemiological factor in the appearance of epidemics [23]. Taken together, these elements suggested that "older echovirus strains" could be recycled and gave onset to new out breaks after successive distant transportation. However, echovirus and EV phylogenetic evolution remained incompletely understood because of unsampled asymptomatic or paucisymptomatic infections. The inconstancy of clinical symptoms we reported here has also been evidenced in another recent French study [24].

Concerning hospitalization length, two assertions can be made from our data. First, cases involving children of less than 15 years without comorbidity had a significantly shorter hospitalization length than adult or neonates and children with comorbidity. This assertion is fully in concordance with previous work from Archimbaud and colleagues [7], where children had also a significantly lower hospitalization length than adult or infants despite identical EV-RT PCR turnaround times. Interestingly, in this study where EV-RT PCR was performed daily from Monday to Friday [7], mean hospital-

ization lengths of children and adults were very close to median hospitalization lengths of children and adults reported here where EV-RT PCR was performed three times a week: 1.9 versus 2 days for children and 4 versus 4 days for adults. In this study, this difference between children and adults was also related to the higher rates of discharge from hospital before EV RT-PCR results among children than among adults. Such a practice is probably related to the absence of differential diagnosis among children in case of aseptic meningitis [25], whereas aseptic meningitis due to others viruses or inflammatory diseases can occur in adult patients [2, 25]. This absence of differential diagnosis among children may strengthen the feeling of physician that early discharge of patient with symptomatic treatment will not be pejorative, once the estimation of the probability of Bacterial meningitis has been lowered by clinical examination data, Procalcitonin, Cerebrospinal Fluid lymphocytic predominance... The absence of reduction of hospitalization length as the outbreak was ongoing Fig. (1C) suggested that the age and the absence of comorbidity reduced more the hospitalization length than a memorization bias of the physician about the presence of an ongoing outbreak. Secondly, during this outbreak without diagnostic and therapeutic strategies modifications, the correlation between hospitalization length and EV-RT PCR turnaround time was poor especially in adult patients even in the absence of postlumbar puncture intracranial hypotension (Table 1). This suggested that others subjective determinants of hospital discharge decision probably existed in case of aseptic meningitis (e.g. senior house officers' apprehension to discharge patient from hospital...).

Taken altogether, our findings suggested that (i) EV RT-PCR turnaround time was not correlated to hospitalization length during short outbreaks but only during longer periods [7, 22] (ii) implementation of more expensive daily or Point of Care rapid EV RT-PCR or multiplex neurotropic viral assays only during short or unusual outbreaks could have limited impact onto a significant decrease of hospitalization length and costs.

#### LIST OF ABBREVIATIONS

EV	=	Enterovirus
RT-PCR	=	Real Time-Polymerase Chain Reaction
CSF	=	Cerebrospinal fluid
VP1	=	Viral protein 1
3D-Pol	=	3D Polymerase

#### ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Hospital Ethics Committee approved this retrospective study.

#### HUMAN AND ANIMAL RIGHTS

No Animals were used in this research. All human research procedures followed were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

#### CONSENT FOR PUBLICATION

No objection was raised by any of the study patients in accordance with French Legislation (Loi Jarde).

#### CONFLICT OF INTEREST

The authors report no relationships that could be construed as a conflict of interest.

#### ACKNOWLEDGEMENTS

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YNG took care of the patients, analyzed data and wrote the manuscript, ALL and PAG performed phylogenetic analyses and critically reviewed the manuscript, PS collected clinical data and critically reviewed the manuscript, CP and NB critically reviewed the manuscript, PO took care of the patients and critically reviewed the manuscript, LA validated virological analyses, analyzed data and wrote the manuscript.

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