

## Outbreak

# Molecular diagnosis of echovirus 30 as the etiological agent in an outbreak of aseptic meningitis in Panama: May – June 2008

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## Abstract

**Introduction:** Aseptic meningitis outbreaks are commonly caused by viral pathogens with enterovirus a common etiological agent. Between May and June of 2008, an outbreak of 173 cases of aseptic meningitis occurred in the Chiriqui Province of Panama. Molecular techniques were used to identify the etiological agent.

**Methodology:** Cerebrospinal fluid (CSF) samples from 75 patients were received at the Gorgas Memorial Institute for Health Studies. RNA extraction and one-step RT-PCR were performed on each sample to determine the presence of enterovirus. Thirty-four samples which were positive for enterovirus were subject to group-specific PCR, sequencing, and phylogenetic analysis to identify the etiological agent of the outbreak.

**Results:** The CSF of 58 subjects was found positive for the enterovirus family using RT-PCR. Thirty-four samples were found to belong to the enterovirus B group. Phylogenetic analysis of four successfully sequenced samples revealed echovirus 30 as the etiological agent.

**Conclusion:** Echovirus 30 is reported as the likely cause of an outbreak of aseptic meningitis in Panama, the first since the 1980s.

**Key words:** meningitis, aseptic; enterovirus B, human; echovirus infections; Panama; disease outbreaks; VP1

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## Introduction

The human enteroviruses belong to the *Picornaviridae* family. They are non-enveloped, single-stranded RNA viruses comprising 68 immunologically- and genetically-distinct viruses, classified into four genetic groups, A to D [1]. Transmission of these viruses generally occurs by the respiratory and fecal-oral routes with respiratory transmission predominant in regions of improved hygiene [2]. Infection through contaminated swimming water is also reported [3] and symptoms may be exacerbated by intensive exercise [4]. Epithelial cells of the gastrointestinal and respiratory tracts are primary sites of replication; viremia may follow with infection of secondary organs [2].

Many enterovirus infections are asymptomatic or self-limiting with a non-specific febrile illness or common cold-like symptoms of the upper respiratory tract. Infection may, however, result in more severe illnesses such as hemorrhagic conjunctivitis or acute

flaccid paralysis. These viruses predominate in children aged five years or older, individuals with lower socioeconomic status, and males under 20 years of age (2). In recent years, echovirus 30 has been a frequently identified etiological agent in outbreaks of aseptic meningitis throughout the world (5-9).

In 2008, an increase in cases of aseptic meningitis was reported from various regions of Panama, the majority (173 cases) from the province of Chiriquí. This is the second highest record of aseptic meningitis cases in Chiriquí Province since 2002, when 261 cases were reported (Pablo Acosta, personal communication). In the intervening years and in 2001, fewer than 55 cases per year were reported, with an average of 25 cases per year for the years 2001, 2003-2007 (Pablo Acosta, personal communication). Our study aimed to report the causative agent of the aseptic meningitis outbreak in 2008.

## Methodology

### *Sample collection*

Between May and June of 2008 in the Chiriquí Province of Panama, 173 patients showed clinical signs and symptoms indicative of aseptic meningitis. All patients presented to the José Domingo de Obaldía Hospital in David, the capital city of Chiriquí, and cerebrospinal fluid (CSF) samples were collected from 75 patients, representing over 40% of the 173 cases. In this molecular epidemiological study, the CSF samples were subject to chemical and microbiological analyses in David and molecular analysis at the Instituto Conmemorativo Gorgas de Estudios en Salud (ICGES) in Panama City.

### *Molecular analysis*

Molecular analysis was performed by extracting RNA directly from CSF using the QIAamp Viral RNA Mini Kit (Qiagen, Gaithersburg MD, USA), according to the manufacturer's recommendations. Extracted RNA was subjected to RT-PCR directed at the conserved region in the 5' UTR among the enteroviruses using primers EV1/EV2 for the first PCR and EVD2/EVU2 for the nested PCR, as previously described [10]. Samples showing a 197 bp band by agarose gel electrophoresis were considered positive for enterovirus. To identify the viral species, a hemi-nested PCR was performed using primers to the VP1 region of the enterovirus genome, as previously described [10], and products were visualized on a 1.5% agarose gel. To identify the specific enterovirus responsible for the meningitis outbreak, we sequenced PCR amplicons with the same primers used in the hemi-nested PCR [10].

### *Case definitions*

Level I patients were defined as confirmed cases, displaying both clinical signs and microbiological evidence of aseptic meningitis. This group included 58 (of 75) cases of samples positive for enterovirus by PCR, 34 (of 58) positive specifically to enterovirus B by PCR, and 4 (of 34) samples sequenced.

Level II patients were defined as probable cases, displaying only clinical signs of aseptic meningitis. This group included 17 (of 75 received at Gorgas Memorial Institute) samples testing negative for enterovirus by PCR, and 98 (of 173) samples from which CSF samples were not collected at José Domingo de Obaldía Hospital in David.

### *Phylogenetic analysis*

We performed two phylogenetic analyses. The first analyzed the type of enterovirus involved in the outbreak and the second classified it in the respective lineage. For each analysis, multiple sequence alignments using the samples sequenced in this study and reference strains were performed using CLUSTAL W and Molecular Evolutionary Genetics Analysis Version 4.0 (MEGA 4) software (MEGA, Tempe, AZ, USA). The maximum composite likelihood method was used to evaluate genetic distances. MEGA 4 software was used for pairwise comparisons and to construct neighbor-joining (NJ) trees. The reliability of the NJ tree was estimated by bootstrap analysis with 1,000 pseudoreplicates.

## Results

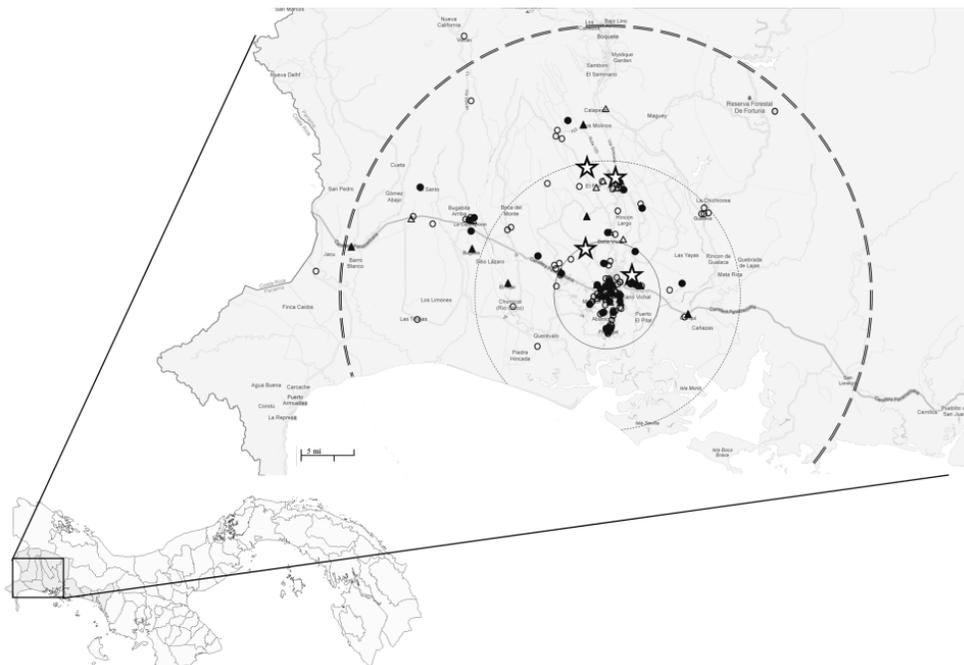
### *Epidemiological and laboratory results*

Between 5 May 2008 and 6 June 2008, a season noted for increasing rainfall and humidity in Panama, an increase in meningitis cases among children was observed, with 173 cases reported. All cases were concentrated in Chiriquí Province, which is located in the north-west of Panama (Figure 1). The total population of this province is 368,790 with 118,504 children younger than 14 years old. We received samples from 75 patients of the total group ( $n = 173$ ) who showed clinical evidence of aseptic meningitis and whose CSF showed no bacterial growth after 72 hours' culture in enrichment media. Ages of the affected patients ranged between 6 months to 13 years and 57% of the patients were older than five years. Glucose concentration in CSF was within normal range for all patients, suggesting a non-bacterial cause of the meningitis. Also, the differential white blood cell counts of CSF showed elevated neutrophils and leukocytes, indicating an infectious cause of the meningitis (Table). A slightly higher incidence of nuchal rigidity in boys was observed, as well as higher leukocyte counts in the CSF in boys under the age of five compared to girls of the same age.

### *Molecular biology results*

In total, 58 of 75 samples (77%) were positive for enterovirus. Viral species were determined for 34 of these 58 enterovirus-positive samples (59%), and all were found to belong to the human enterovirus B species. Four specimens were sequenced and data was submitted to NCBI (accession numbers HM854943-HM854946). A phylogenetic analysis showed that the

**Figure 1.** Geographic location of an outbreak of aseptic meningitis in Panama, 2008



Chiriquí Province is highlighted in the inset and by darker shading on the main map. Stars: sequenced samples; open circles: samples NOT received by the ICGES laboratory; open triangles: samples negative for any type of Enterovirus by PCR; solid triangles: samples positive to Enterovirus by PCR; solid circles: Enterovirus B positive by PCR.

**Table.** Demographic and clinical data for the 58 patients positive for *Enterovirus* by molecular methods

Age	Gender	n	Headache ("yes") n (%)	Vomiting ("yes") n (%)	Fever ("yes") n (%)	Nuchal rigidity ("yes") n (%)	Average leukocyte count (cells/mm <sup>3</sup> )	Whole blood differential count (% neutrophils)**	Average glucose in CSF (mg/dL)	Average leukocytes in CSF (cells/mm <sup>3</sup> )
0-2	M	2	1 (50)	2 (100)	2 (100)	1 (50)	15900	76	60	95
	F	2	1 (50)	2 (100)	2 (100)	1 (50)	16900	83	54	42
3-5	M	14	14 (100)	14 (100)	14 (100)	4 (29)	12979	87	62	79
	F	7	7 (100)	7 (100)	7 (100)	1 (14)	11128	76	64	35
0 - 5	M	16	15(94)	16 (100)	16 (100)	5 (31)	13344	86	62	81
	F	9	8 (89)	9 (100)	9 (100)	2 (22)	12411	77	62	37
6-10	M	17	17(100)	16 (94)	17 (100)	4 (24)	16188	87	68	56
	F	12	12(100)	11 (92)	11 (92)	2 (17)	14875	83	60	70
10+*	M	4	3 (75)	3 (75)	4 (100)	3 (75)	12050	87	68	111
6 - 10+	M	21	20(95)	19 (91)	21 (100)	7 (33)	15400	87	60	66
	F	12	12(100)	11 (92)	11 (92)	2 (17)	14875	83	68	70
<b>Total male</b>		<b>37</b>	<b>35(95)</b>	<b>35 (95)</b>	<b>37 (100)</b>	<b>12 (32)</b>	<b>14511</b>	<b>86</b>	<b>66</b>	<b>73</b>
<b>Total female</b>		<b>21</b>	<b>20(95)</b>	<b>20 (95)</b>	<b>20 (95)</b>	<b>4 (19)</b>	<b>13819</b>	<b>81</b>	<b>56</b>	<b>56</b>
<b>Total</b>		<b>58</b>	<b>55 (95)</b>	<b>55 (95)</b>	<b>57 (98)</b>	<b>16 (28)</b>	<b>14260</b>	<b>84</b>	<b>64</b>	<b>67</b>

CSF: cerebrospinal fluid

\* No female patients observed in this age rang

\*\* 100 x [Number of neutrophils/(Number of neutrophils + number of lymphocytes)]

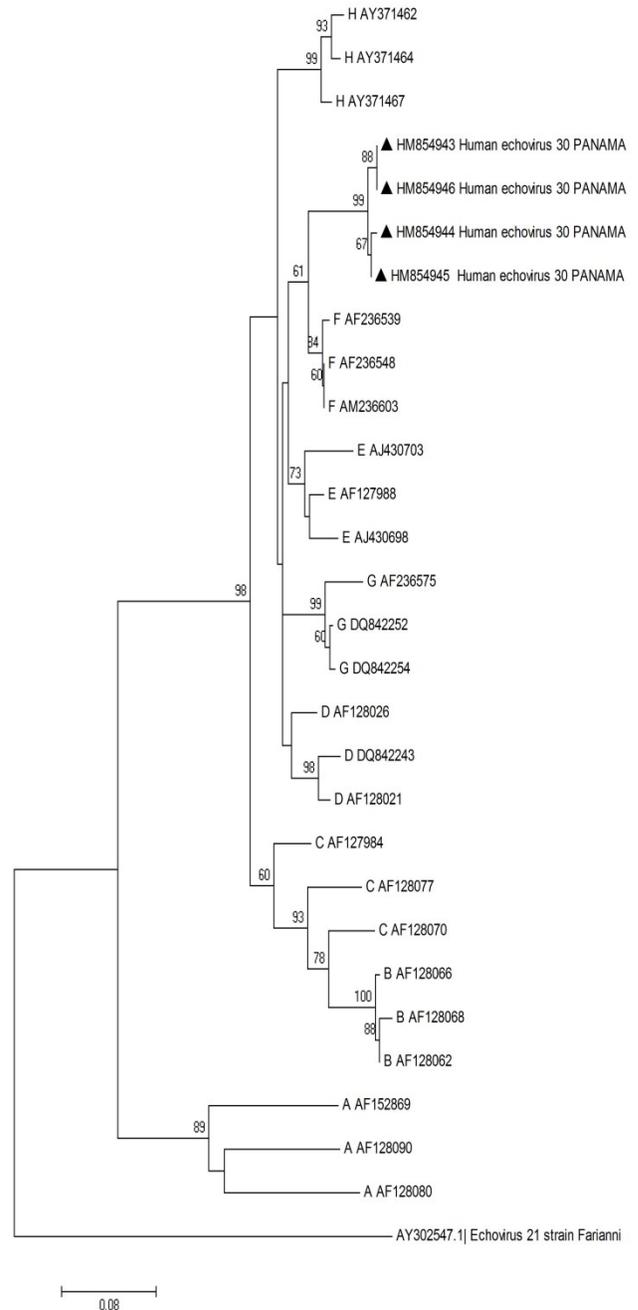
four sequences belonged to the echovirus 30 group of the human enterovirus B species. Analysis of the echovirus 30 lineages showed that the Panamanian viruses were most similar to viruses isolated in Finland and France in 1996 and France in 2005, designated to the F lineage by the nomenclature of Bailly *et al.* [11] (Figure 2).

**Discussion**

The purpose of this epidemiologic study was to identify the causative agent of the outbreak of aseptic meningitis in a specific geographic region of Panama. Our data indicate that human enterovirus B species was isolated in at least 30% of the cases (Level I, confirmed) of the outbreak observed in Chiriquí during May and June 2008, and more specifically that echovirus 30 was implicated in at least four cases. The remaining cases, classified as Level II, showed only clinical signs of aseptic meningitis and we cannot wholly discount other infectious agents being present and the cause of illness in these patients, or in the patients for which CSF tested negative for enterovirus. As reported in other enterovirus outbreaks involving patients aged under 20 years [12,13], male patients were observed more frequently than female patients (1.8:1), although concurrent echovirus 30 outbreaks in Argentina (2007-2008) and Korea (2008) showed a gender ratio of 1:1 and 1.31:1, respectively. The way by which echovirus 30 spread through the community of Chiriquí is unknown, but 50% of the patients were older than 6 years, implicating common environments, such as schools. Panama’s cohort of patients were of a similar age compared to those reported in Argentina, where a mean age of 4.3 years was observed [7]. In Korea, a median age of 5.8 years was observed for Echovirus 30 patients [8].

The enterovirus B echovirus 30 outbreak observed in Panama during 2008 was not unique to this region. Several other countries reported outbreaks with echovirus 30 during 2008, including Korea (May to October 2008) [8]; Japan (September 2008) [4] and Argentina (2007 – 2008) [7]. One goal of our study was to compare clinical data for the Panamanian outbreak with those from other outbreaks that occurred in 2008. In Panama, nearly 100% of cases presented with headache, vomiting and fever, similar to the outbreaks reported in the outbreaks in Japan and at other sites [4]. However, in Panama, only 28% of patients showed neck stiffness, while in Japan and other locations, at least 60% of patients reported this sign [4]. Hayashi *et al.* [4] note that even in the

**Figure 2.** Phylogenetic tree with Panamanian samples and reference strains



Phylogenetic tree showing the relationship between the Panamanian samples (bold circle) and Echovirus 30 strains available in GenBank. The tree is rooted with Echovirus 21 Farianni strain. Both trees were constructed using the Neighbor-joining method with Bootstrap 1000 replicates and the maximum composite likelihood method was used to evaluate genetic distances in the MEGA v4.1 software.

absence of neck signs, however, meningitis should not be excluded from diagnosis.

Our main goal was a molecular comparison of the Echovirus 30 sequences obtained from Panama with those from other outbreaks. However, no sequence data was reported in the Japanese publication; the echovirus 30 sequences from Korea and Argentina were from the H group and the Panamanian sequences were from the F group. A phylogenetic analysis of Panamanian and Korean samples demonstrated that they clustered in different lineages (data not shown) and a comparison with the echovirus 30 sequences from Argentina was not possible; different parts of the virus genome had been sequenced.

To our best knowledge, this is the first defined outbreak of enterovirus and aseptic meningitis in Panama, and Central America more broadly, since the early 1980s. In that decade, acute fevers and aseptic meningitis were reported in epidemic proportions, affecting 1,023 patients, the majority of whom were less than 15 years of age and resident in Panama City [14]. The causative agent was an enterovirus identified by serological methods as echovirus 4, another member of the human enterovirus B species [14]. In 1981, an outbreak of acute hemorrhagic conjunctivitis was observed in the Colon Province of Panama, with the responsible agent identified as enterovirus 70, a member of the human enterovirus D species [15]. In 2002, another outbreak of aseptic meningitis similar to that described in the present report occurred in Chiriquí Province, but the causative agent of the meningitis was not defined. In this previous outbreak, 261 cases were reported between May and July, compared to the 173 cases observed between May and June of 2008 (Pablo Acosta, personal communication, September 2009). This time of year is significant in Panama for the onset of the rainy season and increasing humidity, and we hypothesize that climatic conditions may have encouraged people to stay indoors, facilitating person-to-person spread of the pathogen.

In the past 40 years, echoviruses have been an important recurring cause of large outbreaks of aseptic meningitis throughout the world [6,9,16], alone or in combination with other enteroviruses [3]. Echovirus 30 undergoes several years of quiescence between outbreaks [6,12], indicating that public health services must anticipate and be prepared to identify and manage outbreaks about once every 10 to 15 years. The molecular epidemiological study presented here demonstrates the presence of an enterovirus in Panama that can act as a causative agent of aseptic meningitis.

The cases of echovirus 30 in Chiriquí Province demonstrate the need for Panama to develop and maintain the appropriate infrastructure to conduct timely diagnosis in situations of severe and widespread outbreaks of aseptic meningitis.

## Conclusion

We have detected an aseptic meningitis outbreak in children where the likely agent was enterovirus type viruses in which echovirus 30 was identified in a group of samples; this is the first reported outbreak of enterovirus in Panama, and possibly Central America, since the 1980s.

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