Whole-genome analysis to describe a Human Adenovirus D8 keratoconjunctivitis outbreak in a tertiary hospital

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Running title: AdV-D8 outbreak by whole-genome sequencing (WGS)

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Abstract (250 words)

Conjunctivitis is a frequent ocular disorder due to the human adenoviruses (HAdVs). Only a few of the 45 types of the HAdV-D species are associated with epidemic keratoconjuntivitis. One of these is HAdV-D8.

A nosocomial outbreak due to HAdV-D8 is rarely described because keratoconjunctivitis are clinically diagnosed and treated without the need to characterize the causative agent. Also, it is difficult to type it at molecular level due to the tediousness of classical typing techniques.

In this work we describe the characterization of an outbreak for HAdV-D8 using the recent whole-genome sequencing method (WGS).

Of the 363 patients attended between July 13 and August 13, 2018, 36 may have acquired intra-hospital conjunctivitis. Eleven from 22 samples from the Virology section were selected for WGS analysis.

WGS results showed that ten out of eleven AdV-D8 strains were closely related. The remaining strain, Case 28 was more related to a public sequence obtained from an outbreak that took place in Germany. WGS results showed that our HAdV-D8 strains had a coefficient of similitude from 89.5% to 94.3%.

Whole-genome sequencing is useful in a clinical setting because it avoids viral culture or specific PCR-sequencing. Sequence reads are publicly available and make it easier to compare which clusters are circulating. In conclusion, whole genome sequencing should play an important role in standard routine to describe viral outbreaks

INTRODUCTION

Conjunctivitis is a frequent ocular disorder observed in clinical practice due to a variety of pathogens including bacteria, viruses or parasites. In regard to viral conjunctivitis, the human adenoviruses (HAdVs) are some of the most often causal agents (1).

HAdVs have been associated with a wide spectrum of diseases concerning respiratory, ocular, gastrointestinal, genitourinary systems and obesity (2). HAdVs are divided phylogenetically into seven species, A through G. HAdV classified in B, C and E species are mainly associated with respiratory diseases, those in A, D, F and G with gastrointestinal disease and those in D and E with ocular diseases (2). HAdV-D is the largest and most rapidly growing HAdV species, and contains viruses associated with epidemic keratoconjuntivitis (EKC), a severe, hyperacute ocular surface infection that usually occurs as an outbreak in schools, swimming pools, hospitals, and many other locations as described in the literature(2, 3). EKC is caused by a few of the 45 types of the HAdV-D species: -D8, -D37, -D53, -D56, -D54, -D64, and D-85 (4, 5).

The report of nosocomial outbreaks due to HAdV-D8 is rarely described because keratoconjunctivitis are clinically diagnosed, and treated without the need to characterize the causative agent. And, in the event, that the etiological agent was detected and the outbreak was defined, it is difficult to type it at molecular level due to the tediousness of classical Adenovirus typing techniques, such as neutralization testing, restriction enzyme analysis (REA) or epitope sequencing.

Currently the classical typing of HAdVs is being replaced by sequencing techniques such as whole-genome sequencing (6). To date 103 unique HAdV genotypes (http://hadvwg.gmu.edu) are recognized. The genome of HAdV-D has a highly conserved part, with a high GC content, and a minority part (less than 10%) hypervariable that allows it to evolve into new genotypes (7).

In this work we describe the characterization of an outbreak for HAdV-D8 that happened in the summer of 2018 at the Ophthalmology Department of a University Hospital in Barcelona, Catalonia, using whole-genome sequencing method.

MATERIAL AND METHODS

Cases. Patients attended at the Ophthalmology Department of a University Hospital (Barcelona) during August, 2018 with a clinical diagnosis of keratoconjunctivitis, this is: red eyes, excessive lacrimation, foreign body sensation, photophobia, redness of the bulbar conjunctiva, chemosis, petechial and subconjunctival haemorrhages.

Samples. Exudates from the bottom of the conjunctival sac, one for patient, collected with one regular FLOQSwab (COPAN Diagnostics, Inc) and introduced in a conical tube filled with 3 ml UTM medium. .

Microbiological methods. All conjunctival exudates were processed for adenovirus antigen detection with use of direct immunofluorescence and viral culture.

For direct immunofluorescence, we used Light Diagnostics™ Adenovirus Antibody (Merck). The specimens were spotted onto glass slides and processed by use of standard techniques. The presence of viral antigen was indicated by the appearance of characteristic intracellular apple-green fluorescence in nuclear, cytoplasmic or both locations. The nuclear fluorescence is uniformly bright whereas cytoplasmic is was very often dotted.

For viral culture, specimens were inoculated into each of 6 cell lines: human fibroblasts (MRC5), human epithelial cells (Hep-2 and A-549), human rhabdomyosarcoma (RD), rhesus monkey kidney (LLCMK2) and African green monkey kidney (VERO) cells. Cultures were incubated for 2 weeks on a roller drum at 35ºC. Viruses were identified on the basis of cytopathic effect in cell cultures and confirmed by staining with specific fluorescein conjugated monoclonal antibodies.

In parallel a real-time PCR qualitative detection of DNA from Adenovirus in clinical samples, using RealCycler ADNV (Progenie Molecular) and the SmartCycler (Cepheid) was done. The DNA extraction was done using the BioRobot EZ1 and EZ1 DPS Virus Kit (QIAgen).

Adenovirus genotyping. PCR reactions were set up in a total volume of 25 µl containing 0.5 µM each oligonucleotide (AdTU7 5'-GCCACCTTCTTCCCCATGGC-3' and AdTU4' 5'-GTAGCGTTGCCGGCCGAGAA-3' for PCR, and AdnU-S' TTCCCCATGGCNCACAACAC and AdnU-A GCCTCGATGACGCCGCGGTG for NESTED-PCR)(Yamin Li *et al*. 2015), 2.5 µl of 10X PCR Buffer with 2 mM $MgCl_2$, 200 µM deoxynucleotide triphosphates, 1.25 U de FastStart™ Taq DNA Polymerase (SigmaAldrich) and 5µl DNA (or 2 µl amplicon at Nested reaction). Amplification both PCR and Nested, were carried out in a Thermal Cycler T100 (BioRad) for a total of 36 cycles. After an initial denaturation step at 94 °C for 10 min, each cycle consisted of denaturation at 94 °C for 1 min, followed by annealing at 50ºC for 1 min and an extension step at 72ºC for 2 min. Finally an extended extension at 72ºC for 7 min was done. The PCR product was analysed on a 2% agarose gel and visualized with Greensafe Premiun (NzyTech under ultraviolet light.

The PCR product was purified with the EXOSAP-IT PCR System (Thermo Fisher Scientific) and sequenced using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), with the same oligonucleotides used for PCR. The sequence reaction was then purified with the AutoSeq G-50 Dye Terminator Removal kit and loaded in the 3500 Series Genetic Analyser (Applied Biosystems). The sequences obtained were analysed by the BioNumerics v.8.0 software (Applied Maths) and introduced to the https://www.rivm.nl/mpf/typingtool/enterovirus/ to obtain the genotype.

Whole-Genome sequencing

Viral DNA was extracted from cell cultures with the DNeasy UltraClean Microbial Kit (QIAgen, Germantown, USA) after concentration with 0.22 µm filter (Ibian Technologies, Zaragoza, Spain). The DNA concentration and quality of extracted DNA was determined by Qubit™dsDNA HS Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) and NanoDrop 1000 Spectrophotometer v3.8 (ThermoFisher Scientific, Waltham, MA, USA). The libraries were prepared using a Nextera XT v.01 kit (Illumina Inc., San Diego, CA, USA) and sequenced on an Illumina NovaSeq 6000 sequencer (Illumina Inc., San Diego, CA, USA) to generate 2 x 150 bp paired-end reads. The sequencing reaction was done at Novogene Europe. The obtained nucleotide sequences were trimmed and analysed in BioNumerics v7.6 (Created by Applied-maths NV. Available from https://www.applied-maths.com). Neighbour-joining method was used for the phylogenetic tree analysis, and showed in a maximum parsimony tree.

RESULTS

On August 14, 2018, the Ophthalmology Department have notified to Infectious Diseases Unit an unexpected increase in the number of patients with suspected epidemic keratoconjunctivitis (EKC), who intervened asking for a list of all patients diagnosed with conjunctivitis in the last month (from 13.07 to 13.8). No case is detected between the clinical staff. Exudate from the bottom of the conjunctival sac from these patients was submitted to the Microbiology Department to make a description of the causal agent.

Of the 363 patients attended at the Ophthalmology Department between July 13 and August 13, 36 may have acquired intra-hospital conjunctivitis (Table 1). The first case diagnosed with conjunctivitis was observed on July 20, which had previously been visited on May 8, for cataracts pathology. The second case took place on August 2, but

the patient had been previously visited on July 22. Two days after the first case. Both patients shared the fencing lamp. But, it is between August 5 and 8, that one peak that included 11 cases make the alert of a possible outbreak (Figure 1). All cases visited through July, just after case 1 shared the fencing lamp. From August 14, the Infectious Diseases Unit get involved in the control of the outbreak and an new 22 cases were found until August 30 (Figure 1).

The 22 samples, arrived at Virology section, were studied by fluorescence and viral culture. In one sample (case 30) a viral culture contamination was detected. Among the remaining 21 samples, 13 grew up in the viral culture for adenovirus, and they were genotyped. The genotyping results showed that in all cases it was HAdV-D8.

In order to confirm that they were the same clone we decided to establish the clonal relationship between the strains by whole-genome sequencing. Nevertheless 2 out of 13 strains were disregarded because the DNA extraction did not meet the requirements of quantity and purity (cases 35 and 36).

The 11 genome sequences analyzed formed a cluster supported by a bootstrap value of 100% in 1000 permutations. Figure 2 shows a Maximum Parsimony Tree to represent phylogenetic relationships among our 11 studied strains together with 6 HAdV-D8 genomes obtained from GenBank and used as non-related strains. In this case the definition of an outbreak has been simple because the isolated strains in our hospital showed values of genetic distance between 100 and 700, except for one case. Case 28 was genetically closer related to two of the strains that we introduced as unrelated to our outbreak (AB448769 and AB448767). These two strains are closely related to each otherwithin an outbreak that took place in Germany (4).

Genomic sequences comparison of the epidemical strains showed between 94.3% and 89.5% of coefficient similitude. This similitude is similar to the HAdV-D8 sequences not related to our outbreak.

DISCUSSION

Keratoconjunctivitis (EKC) is a severe infectious eye disease associated with the HAdV-D8, a DNA virus widely spread everywhere and widely described as the causative agent of epidemic outbreaks (1).

On August 14, 2018, the Infectious Diseases Unit of Sant Pau Hospital was notified an increase of the number of patients who were attended at the Ophtalmological Department, and later developed symptoms of epidemic EKC. This fact conducted an investigation, which identified 36 patients with EKC. Observations in the clinical staffs found that all patients had been sharing the fencing lamp instrument, possible cause of transmission.Previous studies have demonstrated that adenoviruses can persist on environmental surfaces for several weeks (8).

Twenty-two of the 36 cases were suitable to a virological study (which included viral culture with subsequent PCR and sequencing) and 13 of these were positive for HAdV-D8. In order to describe and characterize the outbreak, we decided to use the whole-genome sequencing method.Whole genome sequencing is technically less tedious than REA (restriction enzyme analysis) (11) and has demonstrated sufficient discriminatory power (2, 4, 9, 10).

The HAdV-D8 genome is highly conserved (3) and WGS analysis is more exhaustive than the amplification and sequencing of different regions of the major capsid genes (penton base, hexon, and fiber genes) used also by different authors (3).

In our outbreak, whole-genome sequencing with phylogenetic analysis describe a monophyletic cluster of patients infected with HAdV-D8. This is potentially explained for sharing a fencing lamp. The WGS results showed that the HAdV-D8 strains isolated during the epidemic period had a high coefficient of similitude, but lesser than those

obtained by Hage et al. (4). In that study (4), authors found 23 samples with 99.85% of identity which, were divided into two nodes. Nevertheless, our strains closer related between them than with strains isolated from Germany (KP016741, KP016743), US (KT340056, KT340070) or Japan (AB448767, AB448769). We selected these strains from other studies as strains unrelated to the outbreak. The case 28 strain has a greater distance to the HAdV outbreak strains. This greater genetic distance confirms the idea that a single outlier may also circulate in parallel to the outbreak (4).

The outbreak was determined as closed at the end of August. After that, only three cases of conjunctivitis were arrived at Ophthalmology Emergency Department, one in September (HAdV-D8) , one in December (HAdVB3), as well as another one in November (HAdV-D37) at Emergency Paediatrics.

In conclusion, whole-genome approach has shown us the utility of adenovirus sequencing in a clinical setting. BOur whole-genome approach does not need viral culture (although we did not do it directly from clinical samples because we had previously isolated the virus strain), or specific PCR-sequencing. Also, reads are publicly available making it easier to compare circulating clusters for each site, country, citiey and even hospitals within the same city. In our experience, whole genome sequencing should play a mandatory role in standard routine to describe viral outbreaks

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ADDITIONAL INFORMATION

Competing financial interest: The authors declare no competing financial interest.

Accession Codes: The HAdV-D8 genomic sequences produced in this study have been deposited under the following Bio-Project number PRJNA669467.

261 **Table 1.** Epidemiological data from the 36 cases possible implicated in the epidemical adenoviral conjunctivitis.

Case	Age	Gender	Previously Visits	Diagnostic at previous visits	Data diagnosis conjuctivitis	Adenovirus detection	Treatment
1	63	Male	08.05		20.07	Not performed	Tobradex, Aquoral Forte, SF
2	54	Male	22.07	Irritative conjunctivitis	02.08	Not performed	Hyabak, SF
3	65	Female	05.07/09.07/31.07		05.08	Not performed	Aquoral Forte, Isoptoflucon
4	37	Male	01.06/28.07	Corneal erosion by contact lenses	06.08	Not performed	Tobradex, Thealoz,
5	52	Female	11.06/28.07	Retinal detachment / corneal erosion	06.08	Not performed	Thealoz, SF
6	61	Male	18.07	Retinal detachment	06.08	Not performed	Aquoral Forte, SF
7	76	Female	27.07/01.08		06.08	Not performed	DXM, SF
8	33	Male	28.07	Chemical conjunctivitis	06.08	Not performed	Tobradex
9	33	Female	29-31.07/01.08	Corneal erosion	06.08	Not performed	Thealoz, SF
10	73	Female	03.08	Bacterial conjunctivitis	06.08	Not performed	Hyabak, Icol pomada
11	55	Male	16.07/30.07	Retinal detachment	08.08	Negative	De Icol, Neovis
12	82	Male	30.07		08.08	Not performed	Thealoz, SF
13	72	Female	02.08	Triassic tab	06.08	Not performed	Icol pomada, SF

14	53	Female	04/06.08	Conjunctivitis CE	14.08	Not performed	Thealoz, SF
15	35	Male	06.08	Retinal control	14.08	Not performed	De Icol, SF
16	67	Female	07.08	Control of cryocystectomy	17.08	Negative	Xilin, SF
17	73	Male	31.07	Corneal lesions	17.08	Negative	Thealoz, De Icol
18	55	Female	06.08	Granulomatous anterior uveitis	17.08	Positive	Belcils, SF
19	81	Female	06.08	Glaucoma control	17.08	Positive	De Icol, Hyabak, SF,
20	79	Female	16.07	Retinal control	20.08	Negative	Xilin, SF
21	59	Male	01.08	Vitrectomy control	20.08	Negative	Aquoral Forte, SF
22	87	Female	6.08/14.08	Ophthalmia paresia IIIpc	20.08	Positive	
23	70	Male	08,08/16.08	Retinal control	20.08	Negative	Aquoral Forte, Tobradex, SF
24	24	Female	13.08	Irritative conjunctivitis	22.08	Positive	Aquoral Forte, SF
25	60	Female	14.08	Myopia magna, control	22.08	Positive	Hyabak, SF
26	65	Female	19.08/21.08	QPS	22.08	Negative	Aquoral, SF
27	58	Male	13.08	Retinal detachment, control	23.08	Positive	Neovis, SF
28	84	Male	08.08	eyelid edema	27.08	Positive	De Icol, Thealoz
29	89	Female	14.08/17.08/24.08	corneal erosion	27.08	Positive	De Icol, Azarga, Thealoz

30	69	Male	14.08		28.08	Positive	De Icol, Hyabak, SF
31	81	Female	14.08		28.08	Positive	Neovis, SF
32	56	Male	14.08/21.08	desprendiment vitri/control	29.08	Negative	De Icol, SF
33	74	Male	16.08	iridiotomy	30.08	Positive	Hyabak, SF
34	65	Female	16.08/23.08	iridiotomy, control	30.08	Positive	Thealoz, SF
35	69	Male	20.08	biocular druses	30.08	Positive	Hyabak, SF
36	78	Male	20.08	Retinal detachment, control	30.08	Positive	De Icol

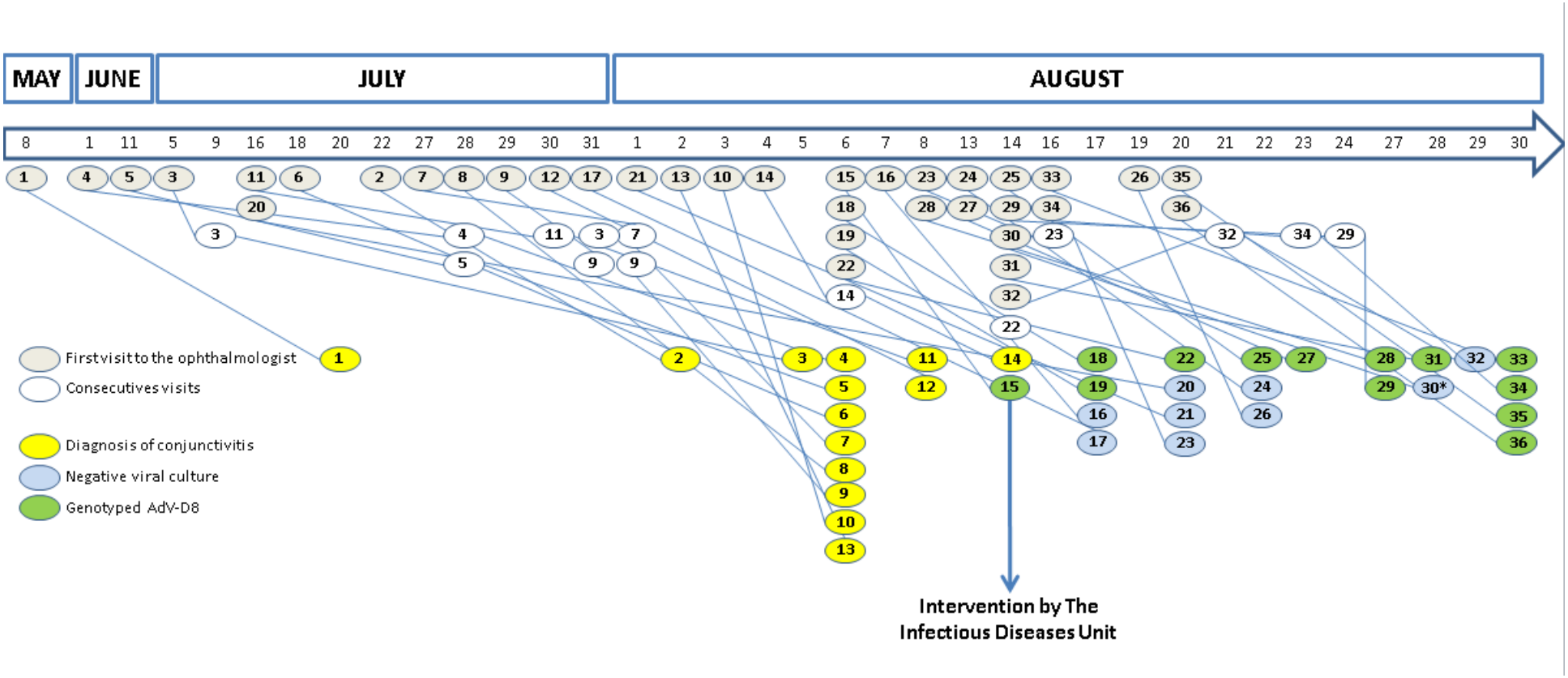

Figure 1. Chronological distribution of the 36 cases with keratoconjunctivitis for AdV-D8 since their first visit to Ophtalmology department and the consecutive ones.

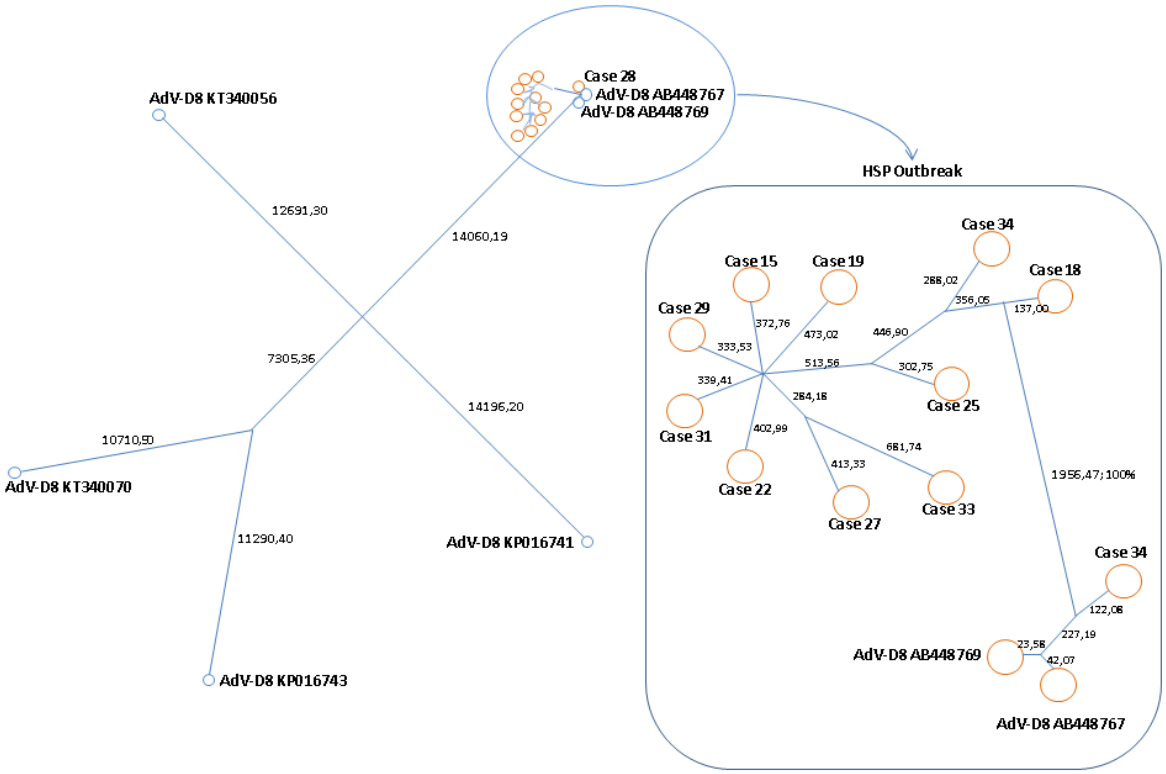

Figure 2. Molecular Phylogenetic analysis by Maximum Parsimony method. The tree was obtained using BioNumerics v.7.6. Bootstrap values (with 1000 permutations) in all cases were 100%.