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ORIGINAL ARTICLE

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Lack of detection of influenza genes in archived formalin-fixed, paraffin wax-embedded brain samples of encephalitis lethargica patients from 1916 to 1920.

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Abstract A method was developed for detection of influenza genes in formalin-fixed brains of mice that had been experimentally infected with influenza A/NWS/33 (H1N1) virus. Using this technique, messenger ribonucleic acid (mRNA) of the β-actin gene was detected in eight clinical brain samples from the 1916–1920 outbreak of encephalitis lethargica, showing preservation of particular mRNAs. However, we did not detect influenza nucleotide sequences of M, NP, and NS genes from these same samples. We conclude either that influenza was not the causative agent of encephalitis lethargica or, possibly, that the virus had a hit-and-run mechanism and was no longer present in the brain at the time of death of the patients.

Keywords Encephalitis lethargica (EL) · Influenza · Gene · PCR · Pandemic

Introduction

The 1918 "Spanish" influenza was the worst infectious pandemic of the last century, infecting half the population of the world and killing 40 million people [12]. Concomitant with the influenza pandemic and extending to the late 1920s, a particular neurological syndrome called Von Economo's disease or encephalitis lethargica (EL) occurred in epidemic proportions. A previous but much smaller epidemic of EL had also coincided with the influenza pandemic of 1889 [5, 6]. On the basis of such epidemiological data, it was suggested that influenza virus was the agent responsible for EL [13].

The polymerase chain reaction (PCR), which allows sequential amplification of a target nucleotide sequence, has revolutionized molecular biology. The sensitivity and specificity of PCR in detecting target sequences has been applied to both fresh and archived formalin-fixed and paraffin wax-embedded tissues [7, 8, 15, 19]. The technique was used to probe eight brain samples, from patients who died of EL between 1916 and 1920, for influenza genes. Our results, which failed to demonstrate the influenza virus genome, cast further doubt on the idea that influenza was the causative agent of encephalitis lethargica.

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Materials and methods

Virus

To provide positive controls for our experiments, mice were infected with a neurovirulent influenza virus, strain A/NWS/33 (H1N1), which should be similar to strains circulating in the 1920s [16]. The virus was grown in eggs and yielded a haemagglutination titre of 196. Ten-fold serial dilutions of virus were made with sterile phosphate-buffered saline A (PBSA) containing penicillin/streptomycin (500 U/ml). For all animal inoculations, fresh dilutions of 1 in 10^3 and 1 in 10^4 were made and kept on ice for no more than 1 h prior to infection.

Inoculation of virus into animals

Three- to four-week-old female Swiss CD1 mice with mean weights of 15–18 g were used in all experiments. All animal inoculations were conducted under light halothane anaesthesia. Each mouse was intracerebrally inoculated in the central frontal region, using a Hamilton syringe and disposable 27-gauge sterile needles, with 20 μl of the designated virus preparation. Mice

Table 1 Main features of post-mortem samples from cases of encephalitis lethargica

Case no.	Year	Sex	Age (years)	Autopsy and cause of death	Histological findings
1	1916	M	3	Clinically, fulminating EL. Brain swelling	Sections of cerebral cortex, brain stem, thoracic cord normal
2	1916	F	Adult	EL. Clinical diagnosis botulism	Perivascular inflammation in the pons and medulla
3	1919	F	44	EL. Brain swelling	Widespread perivascular cuffing, principally in the brain stem and spinal cord
4	1920	M	17	EL. Brain swelling	Meningoencephalitis, principally affecting the brain stem and spinal cord
5	1920	F	37	EL. Rapid loss of consciousness. Died within 24 h	Cerebral oedema and inflammation in the brain stem
6	1920	M	41	Deterioration in conscious level over 1 week. Brain swelling	Inflammation in the upper brain stem
7	1920	M	0.25	EL. Admitted unconscious after a 3-day illness. Died same day	Inflammation in the upper brain stem and spinal cord
8	1920	M	17	EL. Bronchopneumonia and influenza	Extensive inflammation in the brain stem and upper cervical cord

forming the control group were similarly inoculated with 20 μl of sterile PBSA.

Mouse brain samples

Mice were killed 3 days post-infection by asphyxiation with CO_2 according to the UK Home Office guidelines. Each mouse was then immersed in 70% formol saline, the scalp was opened by incision and the brain was removed rapidly using a sterile scalpel and surgical scissors. Brains were chopped finely with scissors and emulsified into sterile plastic universal bottles containing 2 ml of sterile PBSA. Emulsified brain samples were centrifuged at 1,800 rpm for 15 min and RNAs were extracted and stored at −20°C, for use as positive controls in RT-PCR to recover influenza gene fragments.

To ensure good fixation of brains to be wax-embedded, mice were injected with 70% formol saline, which was administered via a tail vein after exposure to carbon dioxide. The formalin-fixed mouse brain was then removed using surgical scissors and placed in a container containing a large volume of 70% formalin prior to paraffin wax embedding 1 week later. Paraffin wax blocks prepared from the brains of mice either infected with A/NWS/33 or inoculated with PBSA were used as positive and negative controls, respectively, for the recovery of influenza gene fragments from human brain samples.

Human brain samples

Eight cases of EL were identified from the autopsy files at the Royal London Hospital for the years 1916–1920 for which formalin-fixed and paraffin wax-embedded brain samples were available in the archives. Outline details are given in Table 1. A paraffin wax block of brain tissue from a patient who had died of Alzheimer's disease in 1996 was used as a negative control. Ten 5 μM sections were cut from each human and mouse tissue block, placed in sealed containers and stored at 4°C until used for RNA extraction.

RNA extraction from tissue sections

Each tissue section was deparaffinized by two extractions with 1 ml *n*-octane on a rotor mixer, with both extractions being performed at room temperature for 60 min. Subsequently, tissues were subjected to two 10-min washes with absolute ethanol, at room temperature, with 2-min centrifugation steps to recover the tissue. Sections were left to air-dry overnight in a fume-hood, with each tube covered by a plastic bag to avoid cross-contamination.

Proteinase K digestion buffer (50 μl 1 M TRIS pH8.0, 2 μl 0.5 M EDTA, 5 μl Tween 20, 20 μl 10 mg/ml proteinase K and 923 μl sterile water) was prepared fresh for all tissue digestions and 100 μl added to each tube containing a deparaffinized tissue section. Tubes were sealed with paraffin film and incubated at either 42°C or 55°C for 3 h, to ascertain the optimal temperature for nucleic acid recovery, then at 99°C for 5 min in a water bath. An empty tube containing only the proteinase K digestion buffer was also processed as a blank/negative control (K1) for each RT-PCR assay. Tubes were centrifuged briefly at the end of the digestion period.

To each digested sample, 225 μl of lysis solution (0.02 M Tris pH7.5, 0.01 M EDTA, 1% SDS) and 25 μl 100 mg/ml proteinase K was added and tubes were incubated at 42°C for 30 min. Samples were then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and twice with equal volumes of chloroform. One-tenth volume of 3 M sodium acetate (pH 5.2) was added to the final aqueous phases with 1 μl of 10 mg/ml oyster glycogen (Sigma) and 2.5 vol of cold absolute ethanol (stored at −20°C) to precipitate RNA. After 20 min on ice, the precipitated RNA was collected by centrifugation at 13,000 rpm for 20 min at 4°C. The pellets were washed with 70% ethanol, air dried, and resuspended in 4.45 μl of sterile water ready for complementary DNA (cDNA) synthesis. A clean-water control (X1) was extracted in parallel with the human and mouse samples.

Oligonucleotide primers

As a test of RNA extraction procedures from human and mouse brains, primers were designed for amplification of β-actin gene and mRNA sequences. Similarly, primers were selected to allow reverse transcription off three genes of the influenza genome which show good conservation between virus strains: NP/segment 5, M/segment 7, and NS/segment 8. For all genes studied, two sets of primers were designed, allowing nested PCR to be performed. A list of primers used is shown in Table 2.

Reverse transcription and PCR

cDNA to viral RNA (vRNA/mRNA) and human β–actin mRNA was synthesized from the RNA extracts using reverse transcriptase (RT, AMV-derived). Reaction mixes (10 μl) contained 1 μl 100 mM

Table 2 Oligonucleotide primers used in RT-PCR gene amplification

Gene	Primer	Direction[a]	Gene position (nucleotide number)	Sequence (5'→3')[b]	Product size (bp)[c]
Human β-actin	BAF 1	F	1284–1307	GAAGAGCTACGAGCTGCCTGACGG	
	BAR 1	R	1840–1863	ATACTCCTGCTTGCTGATCCACAT	373[d]
	BAF 2	F	1338–1361	CTGCCCTGAGGCACTCTTCCAGCC	
	BAR 2	R	1795–1818	GGCCAGGATGGAGCCGCCGATCCA	274[d]
Influenza M (segment 7)	M/32/1	F	32–54	CTTCTAACCGAGGTCGAAACGTA	
	M/273/2	R	250–273	GCATTTTGGACAAAGCGTCTACGC	242
	M/74/1	F	74–96	TCAGGCCCCCTCAAAGCCGAGAT	
	M/190/2	R	166–190	CAGAGGTGACAGGATTGGTCTTGTC	117
Influenza NP (segment 5)	NP/44/1	F	44–68	TCATGGCGTCyCAAGGCACCAAACG	
	NP/326/2	R	301–326	CCTCCAGTyTTCTTxGGrTCyTTCCC	283
	NP/87/1	F	87–110	ACTGrTGGrGAACGCCAGAATGC	
	NP/245/2	R	223–245	ACCATTCTCTCTATTGTkAxGCT	159
Influenza NS (segment 8)	NS/540/1	F	540–562	GAGGATGTCAAAAATGCAATTGG	
	NS/859/2	R	835–859	TAAGCTGAAAxGArAAAGTyCTTAT	320
	NS/566/1	F	566–590	CCTCATCrrAGGACTTGAATGGAAT	
	NS/731/2	R	707–731	CATCTkATyTCTTCrAACTTyTGAC	166

[a] Primers were designed to amplify forward (*F*) to yield positive-sense ssDNA which was back-copied (negative sense) with the reverse (*R*) primer to yield double-stranded DNA
[b] Degeneracy code: y, C/T; k,T/G; r, G/A; w, A/T; x, G/A/T/C
[c] For each gene, two sets of primers were used such that nested PCRs could be performed, the sizes of products using "outer" and "inner" primer pairs are given
[d] The β-actin primers amplify across an intron in the gene so the amplification products off mRNA are 207 bp shorter than off the gene (DNA) template. Primers used for influenza M gene amplification were designed by Dr. J. S. Robertson (NIBSC)

DTT, 2 μl 2 mM dNTP mix (0.5 mM for each dNTP), 1.25 μl RT buffer (0.8 M TRIS pH 8.3 at 42°C, 1.12 M KCl, 80 mM $MgCl_2$), 0.3 μl ribonuclease inhibitor (1 U; CP Labs) and 0.3 μl RT (6 U; Advanced Biotechnologies), with 4.15 μl RNA extract and 1 μl of either 27 μM random hexamers or 10 μM segment-specific primer (for influenza or β–actin sequences). Tubes were incubated at 42°C for 60 min and then boiled for 2 min. A water-only, negative control (X2) was included.

All products of cDNA synthesis were subjected to two rounds of PCR amplification. Each 50 μl PCR mix contained 1 μl cDNA template, 0.25 μM of each forward and reverse oligonucleotide primer, 16 μl dNTP mix (1.25 mM each dNTP), 5 μl reaction buffer (100 mM TRIS pH 8.3, 25 mM $MgCl_2$, 500 mM KCl), and 1 μl (5 U) amplitaq polymerase (Perkin Elmer). Reaction mixes were overlaid with 50 μl of mineral oil and subjected to amplification in a DNA thermal cycler (Perkin Elmer). Each nested reaction used 1 μl of first round PCR product, an inner set of primers and fresh reaction mix. All PCRs were run for 30 cycles of 94°C/30 s, 50°C/1 min, 72°C/1 min. Negative controls, with sterile water as template, were set up in the same manner as the first round and nested PCR (X3 and X4).

First-round and nested PCRs were screened for double-stranded DNA (dsDNA) products by loading 10 μl of each reaction on 2% agarose gels, electrophoresing at 80 V for 60 min, and subsequent staining in ethidium bromide. Samples were run against 0.5 μg of a pUC19/Sau 3A digest (Advanced Biotechnologies) to allow sizing of products, which were visualized and photographed on a UV transilluminator.

Results

Review of archived tissue samples

The sections of brain from the eight archival cases of EL were reviewed. The principal neuropathological finding in all but one case was a florid lymphocytic rhomboencephalitis, without either necrosis or viral inclusions. In four cases the cervical cord was also affected. The findings are summarized in Table 1. Limited material was available for case 1, a 3-year-old male, and, despite having been diagnosed clinically as having EL, this could not be confirmed neuropathologically on the remaining tissue sections. Nevertheless, the autopsy report strongly suggested the diagnosis.

Influenza infected mouse brain: detection of β–actin and influenza gene fragments.

To establish the sensitivity of our technique, we used formalin-fixed brain samples from mice infected with A/NWS/33 (H1N1) [16]. The effects of performing proteinase K digestion at 42°C and 55°C and the use of ribonuclease inhibitor in the RT step were studied.

Following use of random hexamers in the RT step, influenza and β–actin gene-specific primers were used in the PCR steps. For NP-specific primers, a product of 283 bp was detected in extracts of some sections of a mouse brain digested at 55°C whether or not ribonuclease inhibitor was used in the RT step (Fig. 1a: upper block, lanes 1–10). After nested PCR, products of 159 bp were detected for all ten extracts, five of which had been digested at 42°C (Fig. 1a: lower block, lanes 1–10), confirming the expected increase in sensitivity provided by nested reactions. Similar results were obtained when M- and NS-specific primers were used (not shown). This infers a slight advantage when conducting digestion at 55°C. For β–actin, fragments of 373 bp were detected in

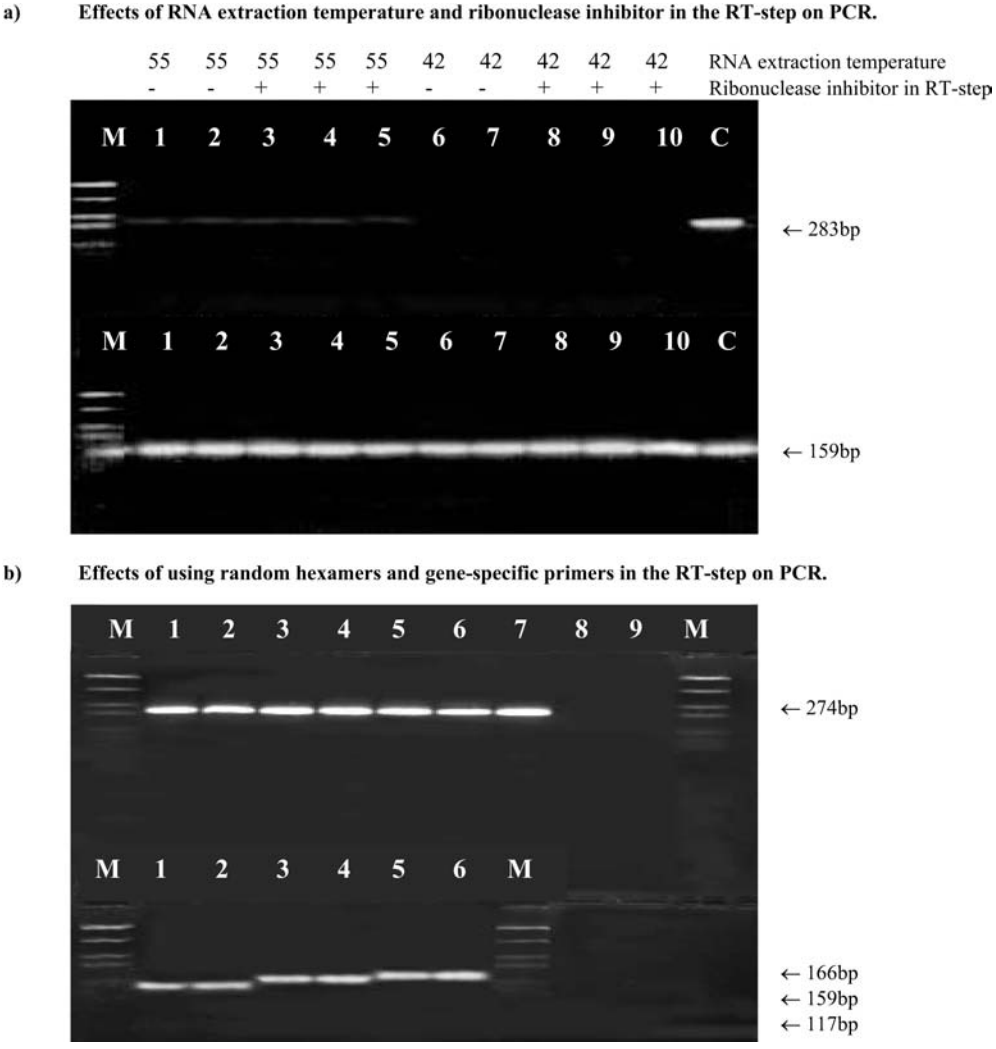

Fig. 1 Recovery of influenza and β-actin gene fragments from extracts of A/NWS/33-infected, paraffin wax-embedded mouse brain. *M* indicates marker lanes (pUC19/Sau3A) and shows bands of known size on a DNA ladder. **a** The temperatures used for RNA extraction from sections of wax-embedded mouse brain and the presence (+) of ribonuclease inhibitor in the RT step are indicated *above the lane numbers*. Random hexamers were used for cDNA synthesis. Influenza NP-specific primers were then used in the PCRs. The *upper block* shows analysis of first round PCR products, whilst the lower block shows results for the nested reactions. *C* indicates a positive control for amplification off an extract of a mouse brain infected with A/NWS/33 which had not undergone formalin-fixation and wax-embedding. **b** *Upper block*: Detection of β-actin gene fragments following use of random hexamers in the RT step. *Lanes 1–6*, six sections of paraffin wax-embedded mouse brain; *lane 7*, a positive PCR control for unfixed influenza virus-infected mouse brain tissue; *lanes 8 and 9*, water negative controls of PCR (*X3, X4*). *Lower block*: Following use of M-, NP- and NS-specific primers in the RT step, extracts of two sections of paraffin wax-embedded infected mouse brain for each, gene-specific nested PCRs were performed. The segment-specific primers used in cDNA synthesis were: M/32/1, lanes 1 and 2; NP/44/1, lanes 3 and 4; NS/540/1, lanes 5 and 6

first-round PCRs (not shown) and 274 bp in the nested reactions (Fig. 1b: upper block, lanes 1–7).

To ascertain whether use of gene specific primers in the RT step might improve the sensitivity of detection, experiments were conducted with influenza gene-specific primers. Again, first-round PCRs were negative (not shown), but nested reactions yielded fragments of 117 bp, 159 bp and 166 bp for M, NP and NS, respectively (Fig. 1b: lower block; lanes 1–6). Overall this indicated no advantage in using gene-specific primers in the RT step, whilst use of random hexamers should provide the opportunity to screen for fragments of different genes in subsequent PCRs. Therefore random hexamers were used for cDNA synthesis in the analyses of extracts of human brain samples.

RT–PCR analysis of human brain samples for the presence of influenza genes

As for the studies of formalin-fixed, wax-embedded brain samples from mice, the EL-related cases (Table 1) and the 1996 Alzheimer's case were all positive for β-actin. Ten sections from each wax-embedded sample were screened

and both rounds of PCR were positive for all sections, with defined product sizes of 373 bp and 274 bp, respectively (not shown).

In contrast, when the same RT products were subjected to nested PCR for influenza genes, M, NP and NS, in no instance were products generated of the expected sizes. In the same PCR sets, all positive controls (coming off influenza A/NWS/33 vRNA template in the RT reactions) were positive, and the negative controls, including K1 and X1–4, were negative (not shown). Overall this demonstrates a lack of influenza gene fragments in any of the archived formalin-fixed wax-embedded human brain samples

Discussion

Here we have assessed the use of RT-PCR in attempting to verify whether or not influenza virus is the causative agent of EL. The mouse model was used as a source of formalin-fixed, wax-embedded brain tissues from control animals and those infected with a neurovirulent strain of influenza, to allow development of a sensitive assay. Using either random hexamers or gene-specific primers in the RT step followed by gene-specific nested PCR, host β-actin mRNA derived gene fragments of defined size were detected in all tissue sections and in both rounds of PCR. Similarly, when either random hexamers or influenza gene-specific primers (for M, NP and NS genes) were used in the RT step and influenza gene-specific primers in PCR, products of defined size were detected in samples derived from A/NWS/33-infected animals but usually after the second (nested) round of PCR only. The latter result probably indicates a lower level of influenza RNA (vRNA/mRNA) in the tissue extracts compared with that of β-actin mRNA.

Random hexamer priming of RT followed by gene-specific nested PCR was applied to RNA extracts of eight human brain tissue sections derived from patients who died of encephalitis lethargica between 1916 and 1920 and another who died of Alzheimer's disease in 1996. Again, for all tissue sections, both rounds of PCR yielded products of defined size for β-actin, but none of the human samples yielded influenza gene-specific products.

A facile explanation for the negative results obtained for the RT-PCR analysis of human archival tissue sections conducted here would be that the influenza gene-specific primers used were incompatible with the infecting influenza. This seems unlikely, as the three genes studied show good sequence conservation between influenza A subtypes. A number of other factors may have contributed to the negative results of the current study.

Firstly, it is possible that the clinical samples either were not infected with influenza virus or that, even if influenza had caused the disease, the mechanism could have been hit-and-run, with only small foci of infection being established. Multiple sections, ten from each human block, were analysed in parallel to minimise false-negative results due to small sample size and possible focal distribution of vRNAs within the tissue block. Secondly, the possibility of RNA degradation during tissue collection and storage may have limited the detection of target sequences. However, we optimized the test by using nested primers which amplified shorter sequences (<180 bp) to increase the sensitivity of detection of target sequences, and β-actin gene fragments of 373 bp and 274 bp were detected. Thirdly, delays between sample collection and formalin fixation after death of the patient may have resulted in loss of RNA through autolysis. This seems unlikely given the sizes of β-actin gene fragments recovered and the fact that the human brain contains low levels of ribonucleases and high levels of ribonuclease inhibitors compared with organs such as the lungs [3, 11]. Fourthly, the titre of influenza in human brain might have been extremely low compared with that which was reached in the lung, thereby making detection more difficult with brain samples. In support of the latter point, isolation of influenza gene sequences from formalin-fixed, wax-embedded lung tissue specimens of two US soldiers who died of primary viral pneumonia during the 1918 influenza pandemic has been reported [19, 14]. More recently, the Royal London Hospital archive has yielded formalin-fixed, wax-embedded lung samples from civilian patients who died of pneumonia during the 1918–1919 pandemic that have yielded influenza haemagglutinin gene sequences (Reid AH, Janczewski TA, Greif R, Elliot AJ, Daniels RS, Berry CL, Oxford JS, Taubenberger JK, unpublished work).

The negative molecular analyses presented here are consistent with those of other workers who failed to detect influenza genes in brain specimens of EL victims [9]. The absence of detection of influenza gene fragments in extracts of human brains derived from EL victims does not rule out influenza virus as a contributory factor in the pathology of these cases. Indeed, most epidemiological evidence supports influenza being the most likely candidate virus to cause EL.

The neurovirulent potential of influenza viruses has been demonstrated by infection of mouse brains and led to an understanding of the underlying mechanisms of viral adaptation to allow growth in neural tissues [21]. Further, in developing a mouse model for Reye's syndrome, RT-PCR detection of influenza gene fragments in a variety of tissues including brain has been reported [1]. Whilst studies in humans have been somewhat limited, cases of influenza-related encephalitis have been reported where cerebrospinal fluid (CSF) has proven either RT-PCR- or isolation-positive for influenza [20, 2, 10, 4]. For an autopsied paediatric case of suspected influenza-induced encephalopathy, immunohistochemical staining identified influenza antigen in meningeal and ependymal areas, neurons of the circumventricular regions, the cerebral and cerebellar cortices and zona compacta of the substantia nigra, whilst RT-PCR analysis indicated infection of limited parts of the brain [18]. In many of these studies, the severity of disease and its presentation has been considered inconsistent with the levels of influenza

detected, and it has been suggested that influenza may act in concert with other infectious agents such as human herpesvirus 6 or 7 [17].

If influenza is a causative agent of EL, then influenza pandemic(s) predicted for the twenty-first century should provide further clinical material for molecular investigations. In the meantime, investigation of the current causes of EL, though diagnoses are rare, could shed light on the wider range of potential causative agents and thereby allow directed re-analysis of the valuable but limited archived samples.

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