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Coronaviruses in brain tissue from patients with multiple sclerosis

Abstract Brain tissue from 25 patients with clinically definite multiple sclerosis (MS) and as controls brain tissue from 36 patients without neurological disease was tested for the presence of human coronaviral RNA. Four PCR assays with primers specific for N-protein of human coronavirus strain 229E and three PCR assays with primers specific for the nucleocapsid protein of human coronavirus strain OC43 were performed. Sporadic positive PCR assays were observed in both patients and controls in some of the PCR assays. However, these results were not reproducible and there was no difference in the proportion of positive signals from the MS patients compared to controls. Evidence for a chronic infection with the human coronaviruses strain 229E or OC43 in brain tissue from patients with MS or controls has not been found in this study.

Keywords Multiple sclerosis · Coronavirus · Polymerase chain reaction · Brain

Introduction

The cause of multiple sclerosis (MS) is so far unknown, but both heredity and environmental factors are considered important. Environmental factors, especially viruses, have been the subject of extensive research and discussion, but no specific agent has so far been definitely linked to MS [12].

Human coronaviruses are a worldwide cause of the common cold, but are usually not associated with more serious disease in humans [17]. The mouse hepatitis virus, a murine coronavirus, causes a MS-like demyelinating disease in the central nervous system of rodents, and this animal model has been used as a model for MS [9, 27].

Evidence for the presence of coronaviral genes in human brain tissue has been found using polymerase chain reaction (PCR) or in situ hybridization [15, 16, 25]. The aim of this study was to examine brain tissue from Danish MS patients for human coronaviral RNA sequences using reverse transcriptase (RT)-PCR.

Materials and methods

Brain tissue was obtained from 25 patients with clinically definite MS from two Danish tissue collections. Most patients had chronic progressive MS for many years. Data on the age at death was available for 20 patients (median 60 years, range 38–83 years). From the 25 patients with clinically definite MS, specimens from areas with macroscopically visible plaques were sampled from the periventricular mostly white matter. All brain specimens were stored at –70°C before and after RNA extraction. For the older tissue collection the year of death ranged from 1966 to 1981, whereas the newer brain collection was from 1987 to 1990.

As controls, brain tissue was obtained from 36 consecutive routine autopsies. Autopsy of the brain was routinely performed if a patient had shown some clinical signs of possible intracerebral pathology prior to death. The pathological reports were reviewed and no patients had a history of neurological disease. The cerebral lesions were previous cerebral infarction in 2 patients and moderate cerebral arteriosclerosis 4 patients. Of the controls 27 had normal cerebral pathology. The causes of death were cancer in 13, lower respiratory tract infection in 9, acute myocardial infarction in 10, and pancreatitis in 1 of the controls. The autopsy report was not available from 3 patients.

As positive control cell culture supernatant fluid containing human coronavirus strain 229E (HCV-229E, ATCC VR-740) and strain OC43 (HCV-OC43, ATCC VR-759) were used. HCV-229E was cultured on L-132 cells (ATCC CCL5) at 37°C and the cell supernatant containing a $TCID_{50}$ (50% tissue culture infective dose) of 10^6/ml was used as positive control. HCV-OC43 was cultured on HRT-18 cells (ATCC CCL244) at 33°C and the cell supernatant containing a $TCID_{50}$ of 10^5/ml was used as positive control.

Extraction of RNA from brain tissue was performed by homogenization in guanidinium thiocyanate buffer followed by ultracentrifugation in a cesium chloride (CsCl) step gradient [22]. The

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extracted RNA was evaluated by absorption spectrophotometry at 260 and 280 nm, and by RT-PCR specific for myelin basic protein (MBP) mRNA. Samples negative for MBP or with an absorption ratio A_{260}/A_{280} lower than 1.6 were excluded from further analysis.

Eight primer pairs were used in the present study (Table 1) and combined in eight different RT-PCR assays (Table 2). The sensitivity of the PCR assays were tested by serial dilutions of the viral supernatants containing HCV-229E and HCV-OC43. All PCR reactions were carried out in 100-μl reaction volume and the concentration of primers, DNA-polymerase (AmpliTag, Perkin Elmer Cetus) and $MgCl_2$ was optimized for each assay. Extensive tables with details of primer combinations and concentrations of reagents are available from the corresponding author on request [6]. For all pre-PCR procedures filter tips and dedicated pipette sets were used.

The PCR products were visualized by UV transillumination following electrophoresis on 1.5% agarose and subsequent Southern blot on Nytran filter. The blot was performed as described, with the exception that the setup was inverted [24]. After overnight transfer, the Nytran filter paper was rinsed briefly in 2× SSC, and nucleic acids were immobilized by baking at 80°C in vacuum for 2 h. Subsequently, the blots were prehybridized at 42°C for 2 h, followed by hybridization overnight with a HCV-229E or HCV-OC43 DNA probe. Stringent washing was performed in SSC buffer (saline sodium citrate) at 70°C. The probes were produced by PCR as described above from the reference strains and labeled with biotin-7-dATP by nick translation (BioNick, Life Technologies, Rockville, USA). Probe detection was performed using the PhotoGene (BioNick) detection kit according to the manufacturer's instructions. A result was registered positive when a band of the expected size was present after hybridization.

Cycle sequencing was performed according to the manufacturer's instructions (Perking Elmer Applied Biosystems, California, USA) with [α-^{33}P]dATP as radioactive label. The reactions were performed according to the manufacturer's instruction. Electrophoresis was run on a polyacrylamide gel. The gels were dried on filter paper and X-ray films were used to visualize the radioactive bands on the dried gel.

Results

A total of 97 specimens from 61 individuals were assayed in 1063 RT-PCR assays (173 specific for MBP mRNA, 416 for HCV-229E and 474 for HCV-OC43). Twenty-one specimens had an A_{260}/A_{280} ratio lower than 1.6, and 4 of these were also negative for MBP. These 21 specimens were excluded, and only the results of the remaining 76 specimens are described. The 76 specimens yielded a mean of 105 μg RNA/g brain tissue, which is slightly lower than the 200–600 μg/g reported in the literature using the same method of extraction [10, 14, 21]. One MS patient was completely excluded as no RNA was detectable in any of the samples extracted. The sensitivity of the assays were in the range of below 10 for the nested PCR assays to 100 Tissue culture infective doses.

Table 1 Primers used in this study specific for the nucleocapsid protein gene of the human coronaviruses and the human myelin protein gene (*MBP* myelin basic protein)

Primer	Sequence (5'–3')	Bases	GenBank acc. no.
	Human coronavirus 229E [23]		J04419
CORO1 [25]	AGGCGCAAGAATTCAGAACCAGAG	498–521	
CORO2 [25]	AGCAGGACTCTGATTACGAGAAGG	806–783	
CORO3 [25]	AACTTTGGAAGTGCAGGTGTTGTG	964–982	
CORO4 [25]	TGGTTCAGCAGTTGCAGGTGAAGT	1262–1242	
CORO9 [7]	CAGGACCCCATAAAGATGC	400–418	
CORO10 [7]	AGAACGAGCAAGACTCTTGG	842–823	
E7 [1]	TCTGCCAAGAGTCTTGCTCG	819–838	
E9 [1]	AGCATAGCAGCTGTTGACGG	1054–1035	
	Human coronavirus OC43 [11]		
CORO5 [1]	CCCAAGCAAACTGCTACCTCTCAG	215–238	
CORO6 [1]	GTAGACTCCGTCAATATCGGTGCC	520–497	
CORO11 [7]	GCAATCCAGTAGTAGAGCGTCC	103–124	
CORO12 [7]	TGACATCAGCCTGGTTACTAGC	548–527	
O7 [1]	GGATGCCACTAAACCTCAGCAAG	820–842	
O9 [1]	GGTGCGAGTTCTGCAAAGATGG	1035–1014	
	Human MBP [26]		
MBP1 [25]	AGAACTGCTCACTACGGCTCCCTG	87–110	X17290
MBP3 [25]	TCCAGAGCGACTATCTCTTCCTCC	356–333	X17289

Table 2 Overview PCR assays performed on the extracted RNA from brain tissue

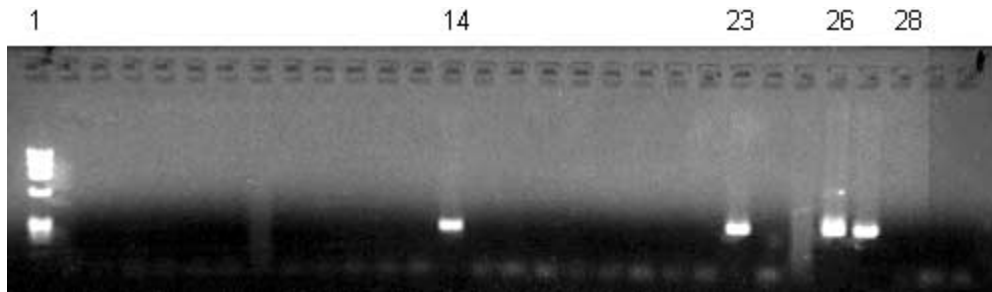| Assay	Forward primers	Reverse primers
MBP	MBP1	MBP3
HCV-229E I	CORO1	CORO2
HCV-229E II	CORO3	CORO4
HCV-229E III (nested)	CORO1,CORO9	CORO2,CORO10
HCV-229E IV	E7	E9
HCV-OC43 V	CORO5	CORO6
HCV-OC43 VI (nested)	CORO5,CORO11	CORO6,CORO12
HCV-OC43 VII	O7	O9

Fig. 1 Results of a nested PCR assay III for HCV-229E. *Lanes 14* and *23* are positive specimens. *Lanes 26* and *27* are positive controls amplified from 3 µl of supernatant fluid with a $TCID_{50}$, of 10^6/ml diluted 10^{-1} and 10^{-3}. *Lanes 28–30* are sterile water controls. The PCR products were of the expected 309 base pairs, were positive on hybridization with the specific probe and yielded the expected DNA sequence.

Table 3 Results of the first specimen tested from each patient. Thus each patient or control is counted only once with the result of one test for comparison. Fishers exact test P=0.55 (*MS* multiple sclerosis)

Number of patients	MS	Controls	Total
Positive in at least one PCR assay	7 (40%)	10 (29%)	17 (32%)
Negative in all PCR assays	12	24	36
Total	19	34	53

Of the specimens tested, 32% were positive in at least one assay. An example of a PCR result is shown in Fig. 1. For comparison between the MS group and controls each individual was only counted once using the first specimen extracted and assay performed (Table 3). In one nested PCR assay for HCV-229E, 31% of the patients were found positive. These results could not be reproduced by repeating the same PCR or on the other PCR analyses, neither on the same or different specimens. These PCR products were sequenced and found identical to the expected sequence from the human coronaviral nucleocapsid gene. There was no proportional difference in these sporadic positive signals between the MS group and controls. No patient or control was consistently positive, neither on reanalysis of RNA from the same extraction or different specimens from the same individual.

Discussion

The yield of RNA extraction was satisfactory, as 93 of 97 specimens contained amplifiable RNA as determined by MBP mRNA RT-PCR assay. The 4 specimens negative for MBP have been excluded as they may either have degraded RNA or contain substances inhibitory to the RT-PCR. A further 17 specimens gave a positive RT-PCR for MBP, but had a low total absorption and a low A_{260}/A_{280} ratio and were thus excluded due to low total RNA content. The remaining 76 specimens had optimal yield and quality. The amount of total RNA in brain tissue should not decrease significantly at least up to 48 h postmortem

[10, 14, 21]; however, some specific mRNA may have shorter half-life [18]. Premortem and postmortem factors may both increase and decrease expression of specific mRNA, but this is mainly important when attempting quantitative RT-PCR [18]. In this study MBP-RNA was amplified from most specimens including the older tissues dating back to 1966. It has been described that storage of RNA at –70°C for more than 5 years may affect the use of RNA for in vitro expression studies but sufficient partial or full-length RNA may remain for PCR studies [14].

The RT-PCR assays yielded scattered positive signals for both HCV-OC43 and HCV-229E. No simple technical explanation can be found for these inconsistent positive signals. One explanation could be a very low level presence of coronaviral target gene in only some of the specimens. If very few (in the order of 10^1 or less) copies of the target gene are present the initiation of amplification in a PCR process becomes problematic. This is due to the probability of a target gene copy being present in aliquot and the probability of the primer annealing to the specific template. A low level presence of coronaviruses in the human brain could be possible after an ordinary episode of common cold due to coronaviruses. In animal models murine coronaviruses have been found to enter the brain of mice, rats and owl monkeys after intranasal inoculation [3, 5, 19, 20] and both a hematogenous spread and direct entry via the olfactory nerve has been found.

False positive results due to contamination cannot be ruled out, but extensive precautions were taken to avoid contamination [13] and UNG carry over prevention was used in some of the assays. The negative controls interspersed between the patient samples were consistently negative.

When comparing the results of the present study with the literature (Table 4) we have found a higher frequency of positives in the control group than previously reported. This could be a bias in the previous studies due to more specimens/assays recorded in the groups of patients with MS. The two studies describing HCV genome in the human brain tissue have both counted all the patients with at least one positive signal, disregarding negative results of repeat specimens [16, 25]. Murray et al. [15] used in situ hybridization techniques and found 52% of the patients with MS positive, but only 11% of the 442 sections examined were positive. In the control group 0.8% of 376 sections were positive. In the positive sections only few cells were positive. This study is comparable to the present study in regards to the paucity of the positive signals observed. Talbot et al. [25] have examined 21 specimens

Table 4 Evidence of coronavirus particles, isolates or genome in brain tissue reported in the literature and in the present study. The virus strains detected are marked with bold and underlined

Year and Reference	Methods: virus strains	MS patients	Controls
		Positive/total	
1980 [4]	Culture: **SD, SK**	2/13	0/13
1992 [15]	*In situ* hybr.: **MHV-A59, SD, OC43**, BCV, 229E,TGEV,HSV	12/21	2/21
1992 [25]	PCR: HCV-OC43, **HCV-229E**	4/11	0/11
Present[a]	PCR: **HCV-OC43, HCV-229E**	7/19	10/34
Crude pooling of data		25/64	12/79

[a]Data from the present study from Table 3. Combination of the tables using weighted mean and 95% confidence intervals for the pooled differences: 26%, 12–40%, $P<0.001$ [2]

from 11 patients with MS and 11 specimens from 11 controls. Not all samples from the positive patients were found positive for HCV-229E. However, the results from the positive specimens were reproducible in contrast to the present study. Weighted pooling of the data (Table 4) shows that more patients with MS were positive compared to controls. This estimate could be biased, as negative studies are less likely to be published [8].

The present results do not provide evidence for a chronic infection with the human coronaviruses strain 229E or OC43 in brain tissue from patients with MS or controls. However, few positive signals were found, which could be sporadic contamination, but low level viral expression of in brain tissue cannot be ruled out.

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