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Coronaviruses in spinal fluid of patients with acute monosymptomatic optic neuritis

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Acute monosymptomatic optic neuritis (AMON) may be an initial symptom of multiple sclerosis (MS). Coronaviruses have been implicated in the etiology of MS. The objective of the present study was to look for coronaviral RNA in AMON, which could be present in the initial stages of the development of MS. *Material and methods* – Spinal fluids from 37 patients with AMON and 15 surgical control patients with protrusion of the intervertebral disk were assayed with a nested multiplex polymerase chain reaction with primers specific for human coronaviruses strain (HCV) 229E and OC43. *Results* – Four patients and 1 control were positive for HCV-229E. No evidence of HCV-OC43 was found. The frequency of positive samples was low and there was no statistical difference between AMON and controls. *Conclusion* – This study does not provide evidence for an etiological role of human coronaviruses in acute monosymptomatic optic neuritis.

Key words: human coronavirus; optic neuritis; multiple sclerosis; polymerase chain reaction

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Acute monosymptomatic optic neuritis (AMON) may occur as an isolated event, but is often considered an initial symptom of multiple sclerosis (MS). About half of the patients will convert to clinically definite MS within 15 years (1). AMON frequently lasts from a few days to several weeks and may affect one or both eyes. The main symptoms are loss of vision, retrobulbar pain and dyschromatopsia. The presence of human coronaviral RNA has been demonstrated in brain tissue and spinal fluid from patients with clinically definite MS and controls (2–4). To investigate the possibility of an infection with human coronaviruses (HCV) in early MS and as a possible cause of AMON we have analyzed cerebrospinal fluid (CSF) from patients with AMON using reverse transcriptase reaction and the polymerase chain reaction (RT-PCR) applying primers specific for HCV.

Materials and methods

CSF was obtained by lumbar puncture from 37 untreated patients with AMON. None of the patients had other symptoms of MS at the time of lumbar puncture. Thirty-four samples were obtained from 6 to 104 days (median 23 days) after the onset of symptoms. Three patients had 2 attacks of AMON and the samples were obtained in relation to the first episode 205, 182 and 71 days before their second episode of AMON. In 2 patients different eyes were involved and in 1 patient the same eye. Thirteen of the patients have later developed clinically definite MS. CSF obtained from 15 surgical patients with protrusion of the intervertebral discs were used as controls. The median age of patients and controls with AMON was 33 (range 13–57) and 51 (range 35–66) years respectively (median test $P<0.001$). In the AMON group there were 25 female patients and among the controls there were 8 females. In the RT-PCR assay, a pool of 14 HCV negative spinal fluids, from the routine laboratory, were interspersed at every 3 to 4 patient samples and were included throughout the whole procedure starting with the RNA-extraction. Further negative controls, sterile water instead of sample, were added at each enzymatic reaction, starting with one in the RT-reaction ending with 3 in the second PCR with the inner set of nested primers.

As positive controls for the PCR, cell culture supernatant fluids containing HCV strain 229E (ATCC VR-740, $TCID_{50}$ 10^{-5})and HCV strain OC43 ($TCID_{50}$ 10^{-4}, ATCC VR-759) were used. All samples were stored at –80°C until analysis.

A multiplex nested RT-PCR as detailed below was used, using primers specific for both HCV-229E and HCV-OC43 (Table 1).

RNA extraction was performed by lysis of 100 µl spinal fluid or serum by vortexing 10 s in 400 µl GuSCN-buffer (4 mM guanidiniumisothiocyanate, 1 mM dithiothreitol, 10 mM TRIS, pH 6). Isopropanol (500 µl) was added, vortexed briefly and spun in a microcentrifuge at 13,000 ***g*** for 10 min at room temperature. For the serum samples, complete removal of supernatant after the isopropanol precipitation was important to avoid a sticky protein precipitate following the ethanol step. To the pellet 750 µl 70% ethanol was added, the tube was vortexed and precipitated as described and the supernatant was removed.

Reverse transcriptase (RT) reaction was performed by mixing primers and other reagents (GeneAmp RNA PCR Kit, Perkin Elmer Cetus) in a total volume of 20 µl. The concentration of the primers CORO10 and CORO12 was 0.5 µM. The other reagents were added according to the standard protocol included with the kit; 5 mM $MgCl_2$, ×1 PCR buffer II, 1 mM of each nucleotide (dGTP, dATP, dTTP, dCTP), RNase inhibitor 1 U/µl, reverse transcriptase 2.5 U/µl. Incubation was performed at 42°C for 15 min, at 99°C for 5 min and 4°C for less than 1 h.

A multiplex PCR using specific primersets for HCV-229E and HCV-OC43 (Table 1) was performed in a total volume of 100 µl. The final concentration of reagents for PCR including the reagents carried over from the RT-reaction: 0.4 µM of the primers CORO9 and CORO10), 0.2 µM of the primers CORO11 and CORO12, 2.5 mM $MgCl_2$, ×1 PCR buffer II, 1 mM of each nucleotide (dGTP, dUTP, dTTP, dCTP), DNA-polymerase (AmpliTaq, Perkin Elmer Cetus) 0.03 U/µl, uracil-N-glycosylase (Boehringer Mannheim) 0.01 U/µl, 2.5 mM $MgCl_2$. Temperature cycles were 37°C (10 min), 95°C (10 min), then 94° – 58° – 72°C (1 min each, 40 cycles), 72°C (15 min) and finally soaked at –5°C. One µl was transferred to the inner PCR reactions. The inner PCR reactions were performed separately for each virus without repeating the uracil-N-glycosylase step. For HCV-OC43 primers CORO5 and CORO6 with an annealing temperature at 62°C and 2.25 mM $MgCl_2$ were used. For the HCV-229E primers CORO1 and CORO2 an annealing temperature of 68°C and 2.0 mM $MgCl_2$ were used.

Table 1. Primers used in this study specific for the nucleocapsid protein gene of the human coronaviruses. The primers CORO9, CORO10, CORO11 and CORO12 were designed for this study

Primer	Type	Sequence (5'–3')	Bases	Ref.
		Human coronavirus 229E (Genbank no. J04419)		(7)
CORO1	f,i	aggcgcaaga attcagaacc agag	498–521	(2)
CORO2	r,i	agcaggactc tgattacgag aagg	783–806	(2)
CORO9	f,o	caggacccca taaagatgc	400–418	
CORO10	r,o	agaacgagca agactcttgg	823–842	
		Human coronavirus OC43		(11)
CORO5	f,i	cccaagcaaa ctgctacctc tcag	215–238	(2)
CORO6	r,i	gtagactccg tcaatatcgg tgcc	497–520	(2)
CORO11	f,o	gcaatccagt agtagagcgt cc	103–124	
CORO12	r,o	tgacatcagc ctggttacta gc	527–548	

f=forward, r=reverse, i=inner and o=outer primer for the nested PCR.

The sensitivity of the PCR assay was measured by samples containing 10 fold dilutions of the virus containing cell culture supernatants in coronavirus negative spinal fluid. The sensitivity was 10 times $TCID_{50}$ for both HCV-229E and HCV-OC43.

The PCR products were visualized by electrophoresis at 5 V/cm on 1.5% agarose followed by Southern blot on Nytran filter. The blot was performed as described (5), with the exception that the setup was inverted. 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 a HCV-OC43 DNA fragment. These probes were produced by PCR as described above from the reference strains and labelled with biotin-7-dATP by nick-translation (BioNick, Life Technologies, Rockville, USA). Stringent wash was performed in 0.15 SSC for HCV-229E and in 0.2 SSC for HCV-OC43 at 70°C. Probe detection was performed using the PhotoGene detection kit according to the manufacturer's instructions (Life Technologies, Rockville, USA).

The PCR products were sequenced in both directions using primers CORO1 and CORO2 as sequencing primers, cycle sequencing reaction using the primers CORO1 and CORO2 using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Perkin Elmer Cetus, Connecticut, USA). The sequence reactions were separated and analyzed on an Applied Biosystems 373A Automated DNA Sequencer as recommended by the manufacturer. For sequence editing, the Seqed software (Applied Biosystems) was used. Alignment of the sequence results was done using the GCG software package (6).

Table 2. Results of nested RT-PCR for HCV-229E RNA

Patient group	RT-PCR on CSF		Total
	Positive	Negative	
AMON	4 (11%)	33	37
Protrusion of the intervertebral disk	1 (7%)	14	15
Total	5 (10%)	47	52

Results

CSF from 4 patients and 1 control (Table 2) were positive on nested RT-PCR using the HCV-229E primers and all samples were negative with the HCV-OC43 primers. The amplified DNA fragments were only visualized after hybridization and not on the ethidium bromide stained gel, indicating that very few RNA copies had been present in the original patient sample at the limit of the sensitivity of the assay. Ten interspersed negative controls (pooled spinal fluids) included throughout the procedure from RNA extraction to the inner PCR remained negative. Water controls added for each enzymatic step also remained negative.

In the amplified fragments all 261 bases between the primers (Fig. 1) were sequenced. One patient (AMON28) had a frameshift and a base substitution and 1 patient (AMON40) had a base substitution. The control patient (PID375) had an ambiguous sequencing result. Our positive control for HCV-229E and the consensus sequence has 2 base substitutions compared to the published sequence (7) used for the design of the primers, but it is however identical to another available sequence of the same gene (8).

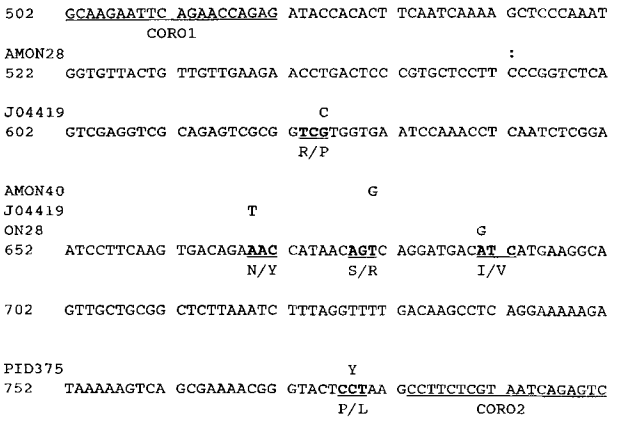

Fig. 1. Results of sequencing of 6 PCR products: 4 patients with optic neuritis, 1 control patient, 1 positive control HCV-229E. Only the consensus sequence and differences are shown (AMON28, AMON40 are patients with optic neuritis, PID375 is the patient with protrusion of the intervertebral disk and J04419 (7) is the published sequence). The bases numbered 502 to 801 are shown. Y=T or C, :=frameshift. The primer sequences CORO1 and CORO2 are underlined. The triplet codons of the reading frame for the nucleocapsid gene corresponding to the differences in base sequence are underlined, and the resulting amino acids are shown below.

Discussion

To our knowledge the presence of human coronaviruses in CSF from patients with AMON has not previously been investigated. In the present study 11% of the patients with AMON and 7% of the controls were positive for HCV-229E and none for HCV-OC43. We consider the possibility of false positive results due to contamination less likely, as uracil-N-glycosylase carry over prevention was used and the negative controls were consistently negative.

On sequencing of the PCR products 2 base substitutions and 1 frameshift mutation were found. Furthermore, 1 control patient had an ambiguous sequencing result. The error rate of PCR using the Taq polymerase has been estimated to be between $1/10^3$ and $1/10^4$ nucleotides or less depending on the initial number of target copies (9, 10) and the reaction conditions. Also the RT step may incorporate errors into the template for the PCR. In this study 6 PCR products of 261 nucleotides equal to 1566 nucleotides were sequenced (Fig. 1) and 4 differences were found. This gives a frequency of 1 mutation per 392 bases. From these few differences cannot be distinguished between true genomic variation and experimental errors in PCR or DNA-sequencing. The nucleocapsid gene of coronaviruses seems well conserved. There is only one published sequence of the ATCC type strain HCV-OC43. Comparison of HCV-OC43 and bovine coronavirus nucleocapsid gene (6, 11, 12) show a 96.6% homology of the coding sequences of 1347 bases, and only 6 differences over the 258 bases between the inner primers amplified in this study. Thus large genomic variation may not be expected, even in strains originating from different mammal species. Concerning HCV-229E there are two published sequences of the nucleocapsid gene from the same ATCC type strain (7, 8). HCV-229E is not as closely related to other known coronaviruses (7), and the natural sequence variation in clinical strains has not been studied.

Another study (4) has also investigated CSF with PCR-technique for HCV. The CSF from patients with MS and controls with other neurological disease were analyzed for the presence of human coronaviral RNA sequences. The study found the CSF from 10 of 20 patients with MS and 9 of 10 controls positive for HCV-OC43. The CSF from 7 of 20 patients with MS and 2 of 10 controls were positive for HCV-229E. Thus in total more than

80% of the tested patients were virus positive. This very high frequency of positive results also in the control is not consistent with the findings in our study. The difference could be explained by differences in sensitivity of the assays or by differences in the selection of the control group. The study by Cristallo et al. (4) does not describe the level of sensitivity, and the control group in the present study consists of patients with protrusion of the intervertebral disk and not of patients with other degenerative neurological diseases.

Conclusion

HCV-229E RNA was found in the CSF by RT-PCR in 4 of 37 patients with AMON and in 1 of 15 controls. The frequency of positive samples was low and there was no statistical difference between AMON and controls. This study does not provide evidence for an etiological role of human coronaviruses in acute monosymptomatic optic neuritis or in early MS.

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