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Metagenomic Analysis of Cerebrospinal Fluid from Patients with Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of central nervous system of unknown etiology. However, some infectious agents have been suggested to play a significant role in its pathogenesis. Next-generation sequencing (NGS) and metagenomics can be employed to characterize microbiome of MS patients and to identify potential causative pathogens. In this study, 12 patients with idiopathic inflammatory demyelinating disorders (IIDD) of the central nervous system were studied: one patient had clinically isolated syndrome, one patient had recurrent optic neuritis, and ten patients had multiple sclerosis (MS). In addition, there was one patient with other non-inflammatory neurological disease. Cerebrospinal fluid (CSF) was sampled from all patients. RNA was extracted from CSF and subjected to a single-primer isothermal amplification followed by

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NGS and comprehensive data analysis. Altogether 441,608,474 reads were obtained and mapped using blastn. In a CSF sample from the patient with clinically isolated syndrome, 11 varicella-zoster virus reads were found. Other than that similar bacterial, fungal, parasitic, and protozoan reads were identified in all samples, indicating a common presence of contamination in metagenomics. In conclusion, we identified varicella zoster virus sequences in one out of the 12 patients with IIDD, which suggests that this virus could be occasionally related to the MS pathogenesis. A widespread bacterial contamination seems inherent to NGS and complicates the interpretation of results.

Keywords

Cerebrospinal fluid • Idiopathic inflammatory demyelinating disorder • Metagenomics • Multiple sclerosis • Next-generation sequencing

1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system of unknown etiology. Epidemiological studies suggest that the development of MS correlates with genetic predispositions and environmental risk factors such as vitamin D insufficiency, cigarette smoking, high estrogen levels, and changes in dietary fats, as well as infections (Pender and Burrows 2014; O'Gorman et al. 2012; Zawada 2012; Kakalacheva et al. 2011; Brahic 2010). The possible role of infections in MS pathogenesis is supported by the uneven worldwide distribution of the disease, its inflammatory character, and human migration studies indicating an increase in disease risk when moving from low to high MS prevalence areas (Ascherio and Munger 2007; Marrie 2004).

In the last decades, over 20 infectious agents (viruses, bacteria, and fungi) have been proposed as a potential cause of MS (O'Gorman et al. 2012; Zawada 2012; Kakalacheva et al. 2011). The relationship between various infections and MS development has been supported by the detection of specific antibodies in the serum and cerebrospinal fluid (CSF), and by the presence of pathogens' nucleic acids and proteins in CSF. The mechanisms explaining how infections might trigger autoreactive immune response include molecular mimicry, viral support of autoreactive cell survival, epitope spreading, and bystander activation (Zawada 2012; Kakalacheva et al. 2011; Brahic 2010).

Several pathogenic candidates have been proposed: Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), human cytomegalovirus (CMV), herpes simplex viruses (HSV) type 1 and 2, human endogenous retrovirus (HERV), measles virus (MeV), or even nonpathogenic torque teno virus (TTV) (Pender and Burrows 2014; Borkosky et al. 2012; Zawada 2012; Zivadinov et al. 2006; Swanborg et al. 2003; Sanders et al. 1996; Norrby et al. 1974). Among bacteria, *Pseudomonas aeruginosa* has been postulated to be involved in MS pathogenesis, as it was shown by Hughes et al. (2001) that specific antibodies are higher in MS patients than in controls. Another study reported that *Chlamydia pneumoniae* IgG antibodies are significantly higher in CSF of MS patients than in control patients and *Chlamydia pneumoniae* DNA has been detected in the CSF and brain of MS patients (Swanborg et al. 2003; Krametter et al. 2001). Other potential pathogenetic candidates underlying MS are fungi whose toxins can be at play in the destruction of astrocytes and oligodendrocytes, leading to myelin degradation (Zawada 2012; Benito-Leon et al. 2010). Despite numerous studies above outlined, there is still no definitive evidence that any particular pathogen is the cause of MS (Brahic 2010).

Most studies on the potential infectious agents in MS concentrate on selected pathogens. There are only few reports that address the microbial flora in MS patients and those that do address it, deal with the intestinal microbiota, especially after the discovery of its role in the dysregulation of innate and adaptive immune response, central nervous system demyelination, and the development of inflammatory bowel disease (Hansen 2015; Joscelyn and Kasper 2014; Round and Mazmanian 2009). The gut microbiome in MS patients has been characterized by a microarray analysis of bacterial 16S ribosomal RNA and changes in the abundance of some taxonomic units, including a lower level of *Faecalibacterium*, have been observed (Cantarel et al. 2015). Miyake et al. (2015) have reported dysbiosis in the structure of gut microbiota in MS patients, compared to healthly controls, consisting of differences in abundances of 21 different species in fecal samples assessed by pyrosequencing. There is still lack of information about bacterial, viral, fungal, and parasitic sequence composition in CSF of MS patients. A new light on potential infectious etiology of MS may be provided by next-generation sequencing (NGS) based metagenomics which enables a simultaneous analysis of numerous microorganisms (Miller et al. 2013; Padmanabhan et al. 2013; Sleator et al. 2008).

In the present study we conducted metagenomic sequencing of cerebrospinal fluids of patients with idiopathic inflammatory demyelinating disorders (IIDD) of the central nervous system, using a single-primer isothermal amplification followed by NGS and comprehensive data analysis (Perlejewski et al. 2015).

2 Methods

2.1 Patients

The study protocol was approved by the Internal Review Board for Medical Research of Warsaw Medical University in Warsaw, Poland. All patients gave written consent for study procedures. The study included 13 patients. There were 12 patients with idiopathic inflammatory demyelinating disorder (IIDD) of the central nervous system; 7 women and 5 men, aged from 22 to 52 years. Ten patients had MS diagnosed on the basis of Polman et al.'s (2011) criteria, one patient had clinically isolated syndrome, and another one had recurrent optic neuritis. In addition, there was one patient with other non-inflammatory neurological disease. CSF was collected through lumbar puncture in all patients during hospitalization at the Department of Neurology of Warsaw Medical University.

2.2 RNA Isolation, Sequencing and Data Analysis

Total RNA was extracted from 500 µl of CSF using the single-step RNA isolation method of Chomczynski (1993) with TRIZOL LS Reagent (Life Technologies; Carlsbad, CA). All samples were elaborated with a single-primer isothermal amplification technique marketed by NuGEN (Ovation RNA-Seq V2; NuGEN, San Carlos, CA). The amplified products were purified using Agencourt AMPure XP beads (Beckman Coulter; Pasadena, CA) and measured with Qubit 2.0 Fluorometer (Life Technology; Carlsbad, CA). Libraries for NGS were prepared using Nextera XT Kit (Illumina; San Diego, CA) following the manufacturer's protocol. In the first step, cDNA was fragmented using transposon-based method and at the same time sequences were marked with indexes by PCR. Subsequently, PCR products were purified with 1.8 volumes of AMPure XP beads (Beckman Coulter; Pasadena, CA). The quality and the length of the sequence library for each sample were measured with a Bioanalyzer (Agilent Technologies; Santa Clara, CA) and either DNA 1000 or DNA HS kit. Finally, samples were pooled equimolarly and sequenced on Illumina HiSeq 1500 (100 nt, paired-end reads).

Raw reads were trimmed by the following procedures: 1/adaptor removal using cutadapt-1.2.1 (Martin 2011); 2/artifact sequence removal using fastx artifact filter; 3/trimming bases with the quality below Q20 (phred quality score) from

3′ end of each read and removing reads shorter than 50 bp using fastq quality trimmer (FASTX-Toolkit 2016). Then, trimmed sequences were mapped onto the human reference sequence (hg19) with the Stampy software (Lunter and Goodson 2011). The unmapped sequences were compared using blastn program against unfiltered NCBI-nt database with e-value cutoff of 1e-5. The taxonomic information of each sequence was assigned and the abundance of identified microorganisms was presented by text mining of blastn output files using BioRuby scripts (Goto et al. 2010).

3 Results

We obtained 441,608,474 reads after sequencing and quality trimming. The highest number of reads was obtained in a CSF sample taken from the patient with other non-inflammatory neurological disease (42,625,952). The number of reads in the IIDD patients ranged from 26,809,197 (Pt. 8) to 40,972,314 (Pt. 9) (Table 1).

Human sequences were the most abundant in all CSF samples from the patients with IIDD (84.35655–97.47609 % of all reads) and in a sample from the patient with other non-inflammatory neurological disease (91.44283 %) (Table 1). In the former samples viral sequences represented 0.00085–0.97591 % of all reads, while in the latter sample they constituted 0.01338 %. Viral sequences detected in the CSF samples obtained from all IIDD patients and from the patient with other non-inflammatory neurological disease matched to bacteriophages. Further, in a sample from the patient with clinically isolated syndrome, 11 reads of varicella-zoster virus (VZV) were found. Bacteria were represented by 0.83873–12.49834 % of reads in samples from IIDD patients and 3.49656 % in a sample from the patient with other non-inflammatory disease. Most abundant bacterial reads mapped to the genomes from *Pseudomonas, Escherichia, Bacillus, Streptococcus, Acinetobacter, Corynebacterium*, and *Moraxella* genera. Fungal reads (0.22931–2.80156 % in samples from the patients with IIDD and 0.40126 % in a sample from the patient with other non-inflammatory disease) represented species from a variety of genera, such as *Malassezia, Ascomycota, Funneliformis, Glomus, Cladosporium, Candida*, and *Alternaria*. Parasites and protozoa constituted 0.04785–0.84515 % of all reads in samples from the patients with IIDD and 0.20770 % of reads in a sample from the patient with other non-inflammatory disease. Representatives of the *Albugo* genus were detected in all investigated samples. Among other parasitic/protozoan reads most mapped to the genomes represented by *Besnoitia, Babesia*, and *Plasmodium* genera. Five most abundant bacterial, fungal, and parasitic/protozoal sequences are shown in Table 2.

4 Discussion

In the present study we demonstrate the results of a metagenomic search for potential infectious agents in CSF of patients with idiopathic inflammatory demyelinating disorder, employing next-generation sequencing. In one of IIDD patients, diagnosed with clinically isolated syndrome, we detected 11 reads which mapped to VZV genome. Finding a DNA virus while analyzing RNA is not unexpected with the methodological approach used. In a previous study we have demonstrated that the Chomczynski RNA extraction, followed by a single-primer isothermal amplification, NGS, and metagenomic data analysis, enables to detect both DNA and RNA sequences (Perlejewski et al. 2015). Interestingly, a relationship between VZV infection and demyelinating disorders has been previously suggested by the demonstration of more frequent presence of VZV-DNA and viral proteins in CSF of MS patients as compared to patients with other neurological diseases or healthy controls (Sotelo et al. 2008; Mancuso et al. 2007). Further, VZV-DNA is more prevalent in CSF and peripheral blood mononuclear cells during MS relapse than in remission (Sotelo et al. 2014; Ordonez et al. 2004).

Table 1 Results of next-generation sequencing (NGS) of cerebrospinal fluid samples from 12 patients with central nervous system idiopathic inflammatory demyelinating disorder (IIDD) and one patient with other non-inflammatory neurological disease. Reads were compared to the NCBI-nt database

Sample ID	Pt. 1	Pt. 2	Pt. 3	Pt. 4	Pt. 5	Pt. 6	Pt. 7	Pt. 8	Pt. 9	Pt. 10	Pt. 11	Pt. 12	Pt. 13
Diagnosis	MS	MS	MS	CIS	RON	MS	MS	MS	MS	MS	MS	MS	OND
Reads after trimming	29,850,818	36,196,465	36,927,824	31,002,606	34,064,072	33,734,539	33,335,714	26,809,197	40,972,314	31,237,666	31,291,530	33,559,777	42,625,952
Human	28,594,910 (95.79272 %)	30,533,365 (84.35455 %)	35,995,798 (97.47609 %)	30,086,680 (97.04565 %)	32,616,258 (95.74973 %)	31,580,310 (93.61417 %)	29,702,485 (89.10109 %)	22,653,060 (84.49735 %)	35,172,506 (85.84457 %)	28,101,524 (89.96038 %)	28,802,337 (92.04515 %)	31,317,498 (93.31855 %)	38,978,376 (91.44283 %)
Viral	1850 (0.00620 %)	1312 (0.00362 %)	1590 (0.00431 %)	263 (0.00085 %)	140,890 (0.41360 %)	2844 (0.00843 %)	2097 (0.00629 %)	10,340 (0.03857 %)	5009 (0.01223 %)	304,852 (0.97591 %)	2453 (0.00784 %)	2671 (0.00796 %)	5702 (0.01338 %)
Bacterial	309,417 (1.03654 %)	4,523,958 (12.49834 %)	309,726 (0.83873 %)	653,442 (2.10770 %)	668,362 (1.96207 %)	569,177 (1.68722 %)	1,771,447 (5.31396 %)	2,364,298 (8.81898 %)	2,730,620 (6.66455 %)	1,954,752 (6.25768 %)	819,745 (2.61970 %)	645,875 (1.92455 %)	1,490,443 (3.49656 %)
Fungal	348,489 (1.16744 %)	301,127 (0.83192 %)	336,543 (0.91135 %)	73,745 (0.23787 %)	78,112 (0.22931 %)	665,311 (1.97220 %)	347,068 (1.04113 %)	237,889 (0.88734 %)	1,022,283 (2.49506 %)	76,744 (0.24568 %)	826,274 (2.64057 %)	940,198 (2.80156 %)	171,043 (0.40126 %)
Archaeal	8 (0.00003 %)	0	6 (0.00002 %)	0	0	473 (0.00140 %)	321 (0.00096 %)	99 (0.00037 %)	102 (0.00025 %)	0	22 (0.00007 %)	18 (0.00005 %)	2057 (0.00483 %)
Parasitic Protozoan	222,058 (0.74389 %)	54,546 (0.15069 %)	108,945 (0.29502 %)	22,349 (0.07209 %)	16,298 (0.04785 %)	278,625 (0.82593 %)	93,886 (0.28164 %)	96,054 (0.35829 %)	139,132 (0.33958 %)	1609 (0.05317 %)	264,459 (0.84515 %)	225,398 (0.67163 %)	88,534 (0.20770 %)
Other*	60,570 (0.20291 %)	213,992 (0.59120 %)	39,762 (0.10767 %)	56,480 (0.18218 %)	468,183 (1.37442 %)	163,876 (0.48578 %)	701,974 (2.10577 %)	863,334 (3.22029 %)	1,012,936 (2.47225 %)	703,031 (2.25059 %)	101,959 (0.32584 %)	84,627 (0.25217 %)	867,090 (2.03418 %)
No match	313,516 (1.05028 %)	568,165 (1.56967 %)	135,454 (0.36681 %)	109,647 (0.35367 %)	75,969 (0.22302 %)	473,923 (1.40486 %)	716,436 (2.14915)	584,123 (2.17882)	889,726 (2.17153 %)	80,154 (0.25659 %)	474,281 (1.51568 %)	343,492 (1.02352 %)	1,022,707 (2.39926 %)

MS multiple sclerosis, *CIS* clinically isolated syndrome, *RON* recurrent optic neuritis, *OND* other non-inflammatory neurological disease
*Sequences related to plants, plant viruses, and synthetic DNA constructs

Table 2 The most frequently identified species/genera in cerebrospinal fluid from 12 patients with Central Nervous System Idiopathic Inflammatory Demyelinating Disorder (IIDD) and one patient with other non-inflammatory neurological disease (OND)

Sample ID	Diagnosis	Viruses*	Bacteria**	Fungi**	Parasites/Protozoa**
Pt. 1	MS		Escherichia (48,060)	Cladosporium (41,876)	Besnoitia (77,858)
			Streptococcus (13,647)	Funneliformis (32,047)	Alexandrium (15,146)
			Staphylococcus (13,309)	Glomus (22,485)	Prorocentrum (9548)
			Salmonella (12,721)	Galactomyces (18,469)	Amphidinium (5908)
			Pseudomonas (10,004)	Rhodotorula (7815)	Plasmodium (5367)
Pt. 2	MS		Acinetobacter (563,147)	Malassezia (90,158)	Albugo (10,690)
			Corynebacterium (368,758)	Ascomycota (28,713)	Strombidinopsis (4458)
			Staphylococcus (269,152)	Pleosporales (13,919)	Pseudoplatyophrya (3229)
			Streptococcus (253,844)	Sclerotium (9855)	Stephanopyxis (2563)
			Actinomycetales (251,931)	Saccharomycetales (7863)	Protostelium (1901)
Pt. 3	MS		Escherichia (59,367)	Cladosporium (38,058)	Besnoitia (37,811)
			Bacillus (36,154)	Galactomyces (28,425)	Alexandrium (5553)
			Streptococcus (18,925)	Funneliformis (15,555)	Albugo (3526)
			Staphylococcus (18,506)	Glomus (11,732)	Stemonitis (3105)
			Micrococcus (14,503)	Candida (9920)	Plasmodium (2842)
Pt. 4	CIS	Varicella-zoster virus (11)	Helicobacter (68,198)	Ascomycota (11,755)	Strombidinopsis (1663)
			Acinetobacter (64,688)	Malassezia (7721)	Albugo (1516)
			Corynebacterium (54,628)	Alternaria (2710)	Euglena (1240)
			Staphylococcus (49,276)	Leptosphaeria (2633)	Nannochloropsis (1232)
			Actinomycetales (30,954)	Zymoseptoria (1858)	Bacillariophyta (1156)
Pt. 5	RON		Corynebacterium (59,747)	Triposporium (4346)	Plasmodium (2760)
			Acinetobacter (49,120)	Mollisina (3997)	Nannochloropsis (2475)
			Bradyrhizobium (39,549)	Podosphaera (3828)	Albugo (2299)
			Micrococcus (39,539)	Melampsora (2973)	Eunotia (1780)
			Klebsiella (29,393)	Pseudogymnoascus (2795)	Babesia (605)

(continued)

Table 2 (continued)

Sample ID	Diagnosis	Viruses*	Bacteria**	Fungi**	Parasites/Protozoa**
Pt. 6	MS		Escherichia (49,987)	Galactomyces (68,600)	Besnoitia (88,141)
			Propionibacterium (40,327)	Funneliformis (43,701)	Stemonitis (10,829)
			Microlunatus (29,628)	Glomus (36,135)	Alexandrium (9832)
			Bacillus (26,898)	Cladosporium (27,492)	Albugo (9047)
			Streptococcus (16,102)	Candida (25,745)	Plasmodium (6520)
Pt. 7	MS		Staphylococcus (471,858)	Knufia (28,791)	Besnoitia (67,537)
			Rothia (226,150)	Funneliformis (25,483)	Stemonitis (1592)
			Pseudomonas (128,641)	Glomus (19,359)	Plasmodium (1045)
			Escherichia (49,170)	Exophiala (10,727)	Polysphondylium (646)
			Lactobacillus (40,061)	Penicillium (7499)	Vermamoeba (628)
Pt. 8	MS		Pseudomonas (922,707)	Funneliformis (20,089)	Besnoitia (62,800)
			Staphylococcus (93,740)	Glomus (14,996)	Albugo (6334)
			Escherichia (84,144)	Malassezia (13,442)	Babesia (2936)
			Stenotrophomonas (61,888)	Debaryomyces (8940)	Stemonitis (1511)
			Corynebacterium (52,014)	Candida (8839)	Plasmodium (1449)
Pt. 9	MS		Pseudomonas (885,872)	Malassezia (199,620)	Besnoitia (72,608)
			Escherichia (160,595)	Brachyalara (34,938)	Amphifilidae (11,494)
			Streptococcus (89,594)	Sclerotium (32,549)	Albugo (7412)
			Rothia (67,661)	Funneliformis (28,706)	Babesia (4581)
			Bacillus (52,881)	Gigaspora (2,2347)	Plasmodium (4580)
Pt. 10	MS		Micrococcus (496,707)	Peniophora (9950)	Plasmodium (3368)
			Lactococcus (119,025)	Malassezia (5551)	Soliformovum (1307)
			Pseudomonas (114,800)	Rhodotorula (3468)	Pylaiella (1154)
			Bradyrhizobium (101,190)	Falciformispora (2664)	Amphifilidae (880)
			Staphylococcus (81,941)	Candida (1637)	Albugo (851)

(continued)

Table 2 (continued)

Sample ID	Diagnosis	Viruses*	Bacteria**	Fungi**	Parasites/Protozoa**
Pt. 11	MS		Kocuria (91,204)	Galactomyces (81,037)	Besnoitia (100,425)
			Streptococcus (43,640)	Funneliformis (52,862)	Alexandrium (10,612)
			Propionibacterium (42,704)	Cladosporium (50,749)	Stemonitis (9079)
			Escherichia (33,717)	Glomus (42,829)	Prorocentrum (6681)
			Micrococcus (31,223)	Candida (28,972)	Albugo (6641)
Pt. 12	MS		Moraxella (107,806)	Galactomyces (102,358)	Besnoitia (104,194)
			Pseudomonas (45,308)	Cladosporium (45,585)	Stemonitis (9481)
			Escherichia (38,946)	Funneliformis (44,676)	Albugo (8374)
			Streptococcus (38,923)	Glomus (36,052)	Polysphondylium (8182)
			Corynebacterium (28,659)	Candida (34,998)	Plasmodium (5422)
Pt. 13	OND		Pseudomonas (259,271)	Funneliformis (20,226)	Besnoitia (59,244)
			Escherichia (161,936)	Glomus (12,842)	Albugo (3081)
			Bacillus (90,784)	Cladosporium (10,331)	Plasmodium (2994)
			Streptococcus (38,627)	Candida (3224)	Babesia (1869)
			Lactobacillus (37,561)	Alternaria (3171)	Bodonidae (1129)

The numbers of sequences representing each species/genera are shown in brackets
MS multiple sclerosis, *CIS* clinically isolated syndrome, *RON* recurrent optic neuritis, *OND* other non-inflammatory neurological disease
*viruses other than bacteriophages; **5 most numerous genera

Although CSF is considered basically sterile, we detected reads that mapped to all the analyzed categories, i.e., viral, bacterial fungal, and parasitic/protozoal. These results are consistent with the observations from other studies which demonstrate a common presence of DNA contamination in metagenomes, which most likely originates from commercial extraction kits and PCR reagents or has an environmental source. The microbial composition depends on the kind of reagents used and its changes occur even on switching from one batch of the same reagent to another one (Weiss et al. 2014). The role of environmental contaminates in metagenomes is also emphasized by the demonstration of different microbial composition in the same sample when analyzed in different facilities (Salter et al. 2014). The low-biomass microbial populations, such as in CSF, seem to be particularly susceptible to contamination in metagenomic studies (Laurence et al. 2014; Salter et al. 2014).

In conclusion, while analyzing CSF samples from 12 patients with idiopathic inflammatory demyelinating disorder we found DNA of varicella zoster virus in one sample. Numerous bacterial, fungal, parasitic, and protozoal sequences were detected in all analyzed samples, which suggests that a widespread contamination, complicating the interpretation of results, is inherent to metagenomic studies.

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