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Increased occurrence of *Treponema* spp. and double-species infections in patients with Alzheimer's disease

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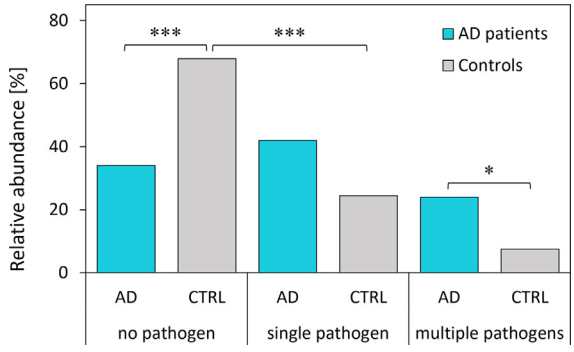
HIGHLIGHTS

- Detection of five bacterial and five viral pathogens in serum and cerebrospinal fluid.
- An increased frequency of Alzheimer's disease patients positive for *Treponema* spp.
- A significantly higher prevalence of cases with two and more simultaneous infections.
- The studied pathogens were widespread equally in serum and cerebrospinal fluid.
- Paralleled analysis of multiple sample specimens provides complementary information.

GRAPHICAL ABSTRACT



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ABSTRACT

Although the link between microbial infections and Alzheimer's disease (AD) has been demonstrated in multiple studies, the involvement of pathogens in the development of AD remains unclear. Here, we investigated the frequency of the 10 most commonly cited viral (HSV-1, EBV, HHV-6, HHV-7, and CMV) and bacterial (*Chlamydia pneumoniae*, *Helicobacter pylori*, *Borrelia burgdorferi*, *Porphyromonas gingivalis*, and *Treponema* spp.) pathogens in serum, cerebrospinal fluid (CSF) and brain tissues of AD patients. We have used an *in-house* multiplex PCR kit for simultaneous detection of five bacterial and five viral pathogens in serum and CSF samples from 50 AD patients and 53 healthy controls (CTRL). We observed a significantly higher frequency rate of AD patients who tested positive for *Treponema* spp. compared to controls (AD: 62.2 %; CTRL: 30.3 %; p-value = 0.007). Furthermore, we confirmed a significantly higher occurrence of cases with two or more simultaneous infections in AD patients compared to controls (AD: 24 %; CTRL 7.5 %; p-value = 0.029). The studied pathogens were detected with comparable frequency in serum and CSF. In contrast, *Borrelia burgdorferi*, human herpesvirus 7,

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and human cytomegalovirus were not detected in any of the studied samples. This study provides further evidence of the association between microbial infections and AD and shows that paralleled analysis of multiple sample specimens provides complementary information and is advisable for future studies.

1. Introduction

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative pathology. It accounts for 60–80 % of dementia cases ("2021 Alzheimer's disease facts and figures," 2021). Dementia is a general term encompassing memory loss and the gradual decline of other cognitive abilities, often serious enough to interfere with daily life and an individual's autonomy. The AD aetiology has not yet been fully elucidated. The most prominent hypothesis for AD pathogenesis is the amyloid cascade hypothesis. This hypothesis states that the most likely cause of AD is a senile plaque formation by the β-amyloid peptide and the generation of neurofibrillary tangles of hyperphosphorylated tau protein (Hardy and Selkoe, 2002). However, the reason for the initial accumulation of β-amyloid is unknown in most patients with AD (Paroni et al., 2019). Kumar and colleagues showed that β-amyloid peptide protects against microbial infection in mouse and worm models of AD (Kumar et al., 2016). Later, Eimer et al. reported that human β-amyloid peptide interacts with herpesvirus surface glycoproteins, accelerating β-amyloid deposition and leading to protective viral entrapment in murine and human AD-like neuronal cells (Eimer et al., 2018). These findings suggest that β-amyloid plaque formation might be induced by an infection. This suggestion is further supported by a growing body of evidence confirming that the β-amyloid is a part of innate immune defence against bacteria, fungi (Soscia et al., 2010; Spitzer et al., 2016), and viruses (Bourgade et al., 2015).

The hypothesis that infection may underpin the aetiology of AD was first raised in 1907 (Alzheimer et al., 1995). Many studies have since described the links between various pathogens and the development of the disease, recently reviewed by Vigasova et al. (2021). A growing body of evidence has associated the infection of different viruses with the risk of developing AD later in life. Examples include the herpes simplex virus 1 (HSV-1) (Jamieson et al., 1991), human herpesvirus 6 and 7 (HHV-6 and HHV-7) (Carbone et al., 2014a; Hemling et al., 2003; Lin et al., 2002; Readhead et al., 2018), human cytomegalovirus (CMV) (Barnes et al., 2015; Carbone et al., 2014b; Lin et al., 2002; Lövheim et al., 2018), Epstein-Barr virus (EBV) (Carbone et al., 2014a), and even SARS-CoV-2 (Chiricosta et al., 2021). Similarly, a variety of bacterial species were implicated in AD pathogenesis, including *Chlamydia pneumoniae* (Balin et al., 2018), *Helicobacter pylori* (Beydoun et al., 2020; Kountouras et al., 2009), *Borrelia burgdorferi* (Miklossy, 2008), *Porphyromonas gingivalis* (Dominy et al., 2019) and *Treponema* spp. (Riviere et al., 2002).

Nevertheless, the concept of the pathogen-induced nature of AD remains controversial despite the growing number of experimental evidence supporting the correlation between infection exposure and AD pathogenesis (Itzhaki et al., 2020; Kayed, 2021). In a recent article, Bocharova et al. reported that the β-amyloid peptide does not protect against herpes simplex virus 1 infection in the mouse brain (Bocharova et al., 2021). Furthermore, Allnutt et al. showed no differences between post-mortem AD and control human brains in terms of viral RNA/DNA load (Allnutt et al., 2020). Although the extent of the causal contribution of infections is not conclusive, certain microorganisms might act as accelerants, exacerbating the disease once it is established (Itzhaki et al., 2020). Evidence suggests that severe sepsis survivors are more likely to develop substantial and persistent cognitive impairment and functional disability (Iwashyna et al., 2010). Moreover, it is unclear whether the disease process involves a single species of microorganism or several ones, and whether they act independently or in combination (Itzhaki et al., 2020). Further research in this field is therefore needed to compare the data collected from multiple specimen types, utilising various sophisticated methods of pathogen detection.

Here we compare the occurrence of the ten most prevalent bacterial and viral pathogens associated with AD. We expected that multiple pathogens could be potentially observed in the samples of AD patients. Using an *in-house* developed multiplex PCR kit, we confirmed an increased occurrence of *Treponema* spp. and double-species infections in serum and CSF of AD patients.

2. Material and methods

2.1. Study participants

We have analysed samples from 50 living AD patients (mean age 71 years; 17 males and 33 females) recruited from the Czech Brain Aging Study (CBAS). The participants presented with mild cognitive impairment (MCI) or dementia stages of the disease (Sheardova et al., 2019) and tested positive for AD biomarkers. The samples collected from these patients consisted of 5 serum samples and 45 serum and CSF matched samples. All CBAS patients underwent a comprehensive diagnostic process including neuropsychological examination, brain MRI, neurological examination and routine blood tests. Control samples were obtained from 53 donors (mean age 45 years; 19 males and 34 females). Serum and CSF samples were obtained from 18 CBAS subjects and 27 cognitively healthy subjects undergoing spinal tap for differential diagnosis of headache or facial palsy. The control set consisted of 9 serum samples, 3 CSF samples and 33 matched CSF and serum samples. To exclude the potential AD pathology, the control subjects were selected with the following criteria: the subjects had negative AD biomarkers in CSF or were APOE 4 negative and cognitively stable for 3 years of follow-up observations. In the cognitively healthy headache and facial palsy group, only those subjects with physiological CSF parameters were included in the study. Eight brain tissue samples were obtained from the patients undergoing surgery for pharmacoresistant epilepsy. All procedures were performed after signing an informed consent form in accordance with the Good Clinical Practice. The name of the Ethics Committee is FN u sv. Anny v Brne and the approval numbers are 45 V/2016 and 46 V/2016-AM.

2.2. DNA isolation and polymerase chain reaction (PCR)

Multiplex PCR kit was developed for detecting five bacterial species (*Chlamydia pneumoniae*, *Helicobacter pylori*, *Borrelia burgdorferi*, *Porphyromonas gingivalis*, *Treponema* spp.) and five viral species (HSV-1, EBV, HHV-6, HHV-7, CMV). 200 μl of thawed serum and CSF samples were used for the isolation of nucleic acids. Brain tissue samples were cut into pieces of a maximum weight of 10 mg. DNA was extracted from these samples using the RTP Pathogen Kit (STRATEC, USA). Each extract was tested for all ten pathogens by amplifying species-specific gene sequences using species-selective primers. All primers used in this study were designed in-house using the Primer-BLAST tool (Table SI 1). Each 20 μl PCR reaction mix contained 2 U of Taq polymerase (Thermo Fisher Scientific, United Kingdom) and 2 U of Uracil-D-glycosylase (New England Biolabs, USA). Conditions of PCR were as follows: initial incubation for 2 min at 37 °C to perform deactivation of old amplicons by Uracil-D-glycosylase, followed by an activation phase lasting 15 min at 95 °C. Samples were then subjected to 45 PCR cycles, each consisting of 5 s denaturation at 95 °C, annealing for 40 s at 60 °C and polymerisation for 20 s at 72 °C. PCR runs were performed in a real-time MIC qPCR cycler (Bio Molecular Systems, Australia). Representative PCR amplification graphs of AD and control samples analysed with *Treponema* spp. assay are shown in supplementary material as Figs. SI 1 and SI 2, respectively. A signal of the specific probe in each pathogen assay was read in the FAM channel (465–510 nm). Conversely, a signal of the tagged internal control sequence was read in the HEX/JOE channel (540–570 nm). Each PCR experiment

included either AD or control DNA, a positive DNA template, and a no-DNA control. A subject was considered positive for a given species if the extract contained a positive signal with Ct ≤ 35.0 within a reaction time as well as a detectable negative signal for negative controls. This threshold was determined by statistical analysis of multiple samples with known composition and successfully validated using the international panels from INSTAND, e.V. (Düsseldorf, Germany; www.instand-ev.de/en/). Positive controls were performed using plasmid vectors carrying loci for respective pathogens. Reagents, negative control samples and disposable supplies were examined for DNA contamination.

2.3. Statistical analysis

Statistical methods used for data analysis included Fisher's exact test, chi-square goodness of fit test and one-sample proportion test. The significance of the difference between AD patients and controls was determined by Fisher's exact test. The chi-square goodness of fit test was used to assess the differences in the distribution of cases with no infection, single infection and multiple infections. Differences between individual groups were further analysed using a one-sample proportion test with Bonferroni correction for multiple testing. All statistical analyses were performed using an open-source software R version 4.0.4 (Giorgi et al., 2022). *p*-values <0.05 were considered statistically significant.

3. Results

3.1. Comparison of bacterial and viral infections between AD patients and controls

To distinguish whether a positive case is infected with single or multiple pathogens simultaneously, we divided all AD patients and controls into four groups. The groups were designed as follows: *group 1* – tested positive for at least one pathogen; *group 2* – tested negative for all studied pathogens; *group 3* – tested positive for a single pathogen; *group 4* – simultaneously tested positive for two or more pathogens. The data from 50 AD patients and 53 controls assigned to *group 1* are shown in Fig. 1. Our results showed a significant difference between AD patients and controls in the prevalence of both bacterial (*p*-value = 0.006) and viral (*p*-value = 0.016) pathogens (Table 1). In *group 1*, almost twice as many AD patients (58 %) were positive for bacterial infection compared to controls (30.2 %). Similarly, the frequency of viral pathogens was 3.5 times higher in AD patients (26 %) than in controls (7.5 %). Our results suggest that gender had no statistical influence on the prevalence of bacterial and viral pathogens across the studied groups. Overall, bacterial pathogens were present in a significantly higher proportion than viral pathogens in both AD patients (*p*-value = 0.006) as well as the control group (*p*-value = 0.016). The *groups 2*, *3* and *4* are compared in Fig. 2. Based on the Chi-square goodness of fit test, the distribution of the groups was equal among AD patients (*p*-value = 0.295), but uneven among the controls (*p*-value < 0.001). The most prevalent group among the controls was *group 2*; having tested negative for all studied pathogens (Table 1). Unsurprisingly, the controls without any pathogen represented 67.9 % of all controls, and their prevalence is significantly higher compared to the 34 % of AD patients in the same group (*p*-value ≤ 0.001). The number of AD patients infected with a single bacterial or viral pathogen (42 %) exceeded the number of controls in *group 3* (24.5 %), however, this difference was not statistically significant (*p*-value = 0.093). Interestingly, simultaneous infection by two or more different pathogens was detected in 24 % of AD patients. This represents a significant prevalence compared to controls where the multiple infections were detected in only 7.5 % of controls (*p*-value = 0.029).

Table 1
Percentages of AD patients and controls tested positive for bacterial or viral infections.

Pathogens		AD patients (*n* = 50)	Controls (*n* = 53)	*p*-values
At least one pathogen	Total	66 %	32.1 %	<0.001
	Bacteria	58 %	30.2 %	0.006
	Viruses	26 %	7.5 %	0.016
No pathogen	Total	34 %	67.9 %	<0.001
Single pathogen	Total	42 %	24.5 %	0.093
Multiple pathogens	Total	24 %	7.5 %	0.029

3.2. Comparison of the prevalence of studied pathogens in serum and CSF

We compared serum and CSF-matched AD patients and controls to explore the prevalence of studied viral and bacterial pathogens in different specimen types. The data from 45 AD patients and 33 controls that tested positive for at least one bacterial or viral pathogen are compared in Fig. 3. Similarly, as in the case with all studied subjects, the results showed a significantly higher prevalence of pathogens among AD patients than in controls (Table SI 2). Pathogens were found with a higher frequency in CSF than in serum, this difference was however not deemed statistically significant (AD: *p*-value = 0.052; CTRL: *p*-value = 0.082). Among the AD patients, 51.1 % had at least one pathogen in CSF, 28.9 % had at least one pathogen in serum, and 17.7 % had at least one pathogen in both CSF and serum. In the control group, 24.2 % of controls tested positive for at least one pathogen in CSF, and 6.1 % tested positive for at least one pathogen in serum. None of the controls tested positive for any pathogen in CSF and serum at the same time.

Fig. 4 shows the relative prevalence of individual bacterial and viral species detected in serum and CSF-matched AD patients and controls. Our results showed that *Treponema* spp. was the most prevalent pathogen among both AD patients (62.2 %) and controls (30.3 %). Moreover, it is the only pathogen found with a significantly higher prevalence in AD patients compared to controls (*p* = 0.007). Although significantly less

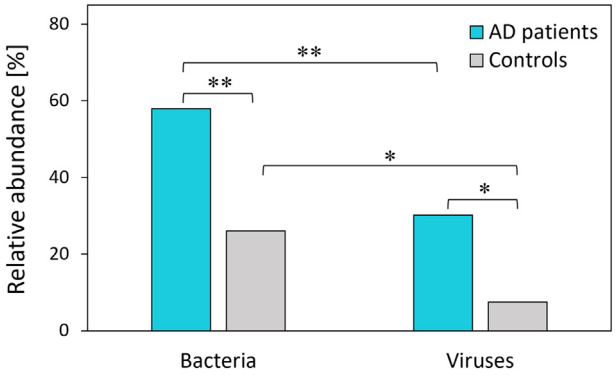

Fig. 1. Percentage of AD patients (turquoise) and controls (grey) with at least one bacterial or viral pathogen detected. Asterisks represent statistical significance (* *p*-value ≤ 0.05; ** *p*-value ≤ 0.01).

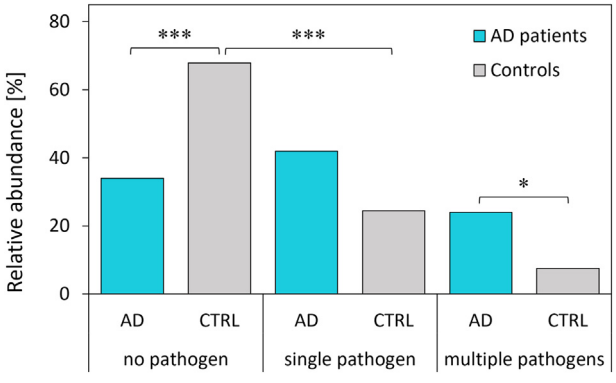

Fig. 2. Number of pathogens simultaneously detected in AD patients (turquoise) and controls (grey). Asterisks represent statistical significance (* *p*-value ≤ 0.05; *** *p*-value ≤ 0.001).

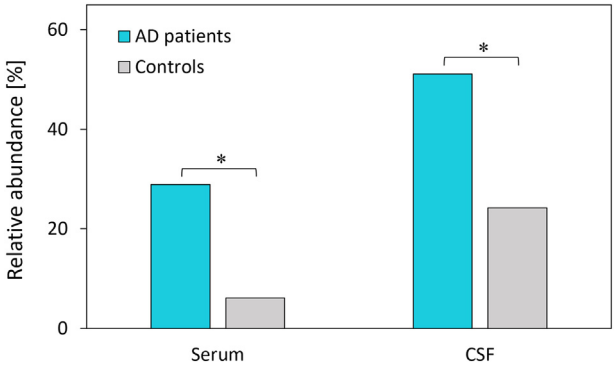

Fig. 3. Comparison of serum and CSF matched AD patients (turquoise) and controls (grey) tested positive for at least one pathogen categorized by specimen type. Asterisks represent statistical significance (* p-value ≤ 0.05).

Table 2
The prevalence of individual pathogens among AD patients and controls.

		AD patients (n = 45)	Controls (n = 33)	p-values
Bacteria	*Treponema* spp.	62.2 %	30.3 %	0.007
	Porphyromonas gingivalis	6.7 %	3.0 %	0.634
	Chlamydia pneumoniae	2.2 %	–[a]	>0.999
	Helicobacter pylori	2.2 %	–[a]	>0.999
	Borrelia burgdorferi	–[a]	–[a]	–[b]
Viruses	HHV-6	15.6 %	6.1 %	0.062
	HSV-1	4.4 %	–[a]	0.506
	EBV	2.2 %	–[a]	>0.999
	HHV-7	–[a]	–[a]	–[b]
	CMV	–[a]	–[a]	–[b]

[a] Pathogens were not detected in these samples.
[b] Not applicable.

prevalent than *Treponema* spp., *Porphyromonas gingivalis* and HHV-6 were also detected in both AD patients and controls. *Porphyromonas gingivalis* was detected in 6.7 % of AD patients and 3.0 % of controls (p-value = 0.634). HHV-6 represents the most prevalent viral pathogen, although its prevalence among AD patients (15.6 %) compared to controls (6.1 %) was not statistically significant (p-value = 0.062). *Borrelia burgdorferi*, HHV-7, and CMV were not detected in either AD patients or the control group. The prevalence of all studied pathogens among AD patients and controls with corresponding p-values is listed in Table 2.

Curiously, there was no significant difference between the frequency of occurrence of the studied pathogens in any specific specimen type. Among AD patients, the presence of *Treponema* spp. was confirmed in 40 % of cases in CSF and 22.2 % of cases in serum (p = 0.110). In the control group, *Treponema* spp. was found in 24.2 % and 6.1 % of CSF and serum samples, respectively (p = 0.082). The prevalence of *Porphyromonas gingivalis* was comparable among AD patients and controls in both serum (AD: 2.2 %; CTRL: 0 %) and CSF (AD: 4.4 %; CTRL: 3.0 %). Notably, HHV-6 was detected at a higher frequency in CSF compared to serum in both AD patients and controls. Among AD patients, it was found in 13.3 % of CSF and 2.2 % of serum samples (p-value = 0.110). The prevalence of HHV-6 in the control group was 6.1 % in CSF and no case was found in serum (p-value = 0.492).

4. Discussion

There is accumulating experimental evidence suggesting the connection between microbial infections and the development of AD (Fülöp et al., 2020; Panza et al., 2019). Until recently, most research in this area has focused on individual pathogens, recently reviewed by Sochocka et al. (2017). However, a growing number of research articles (Table 3) suggest that the aetiology of Alzheimer's disease could be driven by the response to co-infection of multiple pathogens (Vigasova et al., 2021). It is worth noting, however, that almost all these studies used serum pathogen-specific antibodies to identify the infection burden. It therefore could not be determined whether the pathogen-specific antibodies were a response to a current, past, or chronic infection. In this work, we used an in-house PCR kit which allowed for simultaneous detection of five bacterial pathogens (*Borrelia burgdorferi, Chlamydia pneumoniae*, *Helicobacter pylori*, *Porphyromonas gingivalis*, and *Treponema* spp.) and five viral pathogens (CMV, EBV, HHV-6, HHV-7, and HSV-1) in serum and CSF samples. Moreover, previously conducted studies targeted only a single specimen type – serum – whereas parallel analysis of matched serum/CSF carried out in this study increases the relevance of collected data.

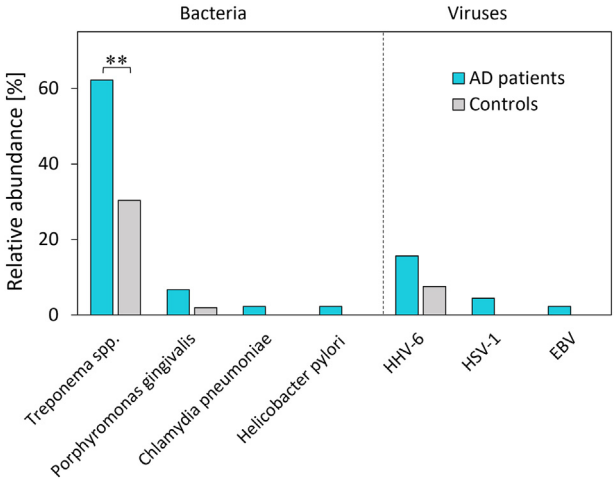

Fig. 4. Prevalence of bacterial and viral pathogens between AD patients (turquoise) and controls (grey). Asterisks represent statistical significance (** p-value ≤ 0.01).

Our results revealed that both bacteria and viruses are significantly more prevalent in AD-affected samples compared to control samples. This observation supports recent studies that confirmed an increased bacterial and viral burden in AD patients (Bu et al., 2015b; Katan et al., 2013; Wright et al., 2015). In contrast, the cumulative viral and bacterial effect on deteriorating cognition was not always supported (Renvoize et al., 1987). Strandberg et al., who tested seropositivity towards HSV-1, HSV-2, CMV, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae* in the elderly Finnish population, observed a positive correlation between viral burden and cognitive impairment; however, no association with bacterial burden was observed (Strandberg et al., 2005, 2003). Conversely, the results of our study showed a significantly higher frequency of bacterial infections than viral infections. This discrepancy could be due to our study's different and broader range of studied bacteria compared to the studies mentioned above. The only significant increase in the prevalence was observed for *Treponema* spp. (p-value = 0.007). No significant difference in the prevalence was found when comparing the serum and CSF. The association between *Treponema* spp. and AD have been previously reported in several studies (Miklossy, 2015; Riviere et al., 2002; Siddiqui et al., 2019). Riviere et al. provided molecular and immunological evidence that oral *Treponema* may infect the brain via branches of the trigeminal nerve (Riviere et al., 2002). Additionally, Kamer et al. reported that peripheral infections can lead to inflammation in the brain and promote β-amyloid deposition (Kamer et al., 2015). Recently, Su et al. confirmed that oral *Treponema denticola* can induce β-amyloid accumulation in the hippocampus of C57BL/6 mice (Su et al., 2021).

Strikingly, all tested samples in this study were negative for *Borrelia burgdorferi*, HHV-7 and CMV, despite these pathogens having been linked to AD in previous studies (Barnes et al., 2015; Herrera-Landero et al., 2019; Lurain et al., 2013; Miklossy et al., 2004; Readhead et al., 2018; Westman et al., 2014). Since we studied serum and CSF samples and *Borrelia burgdorferi* was previously detected in the brain tissue itself

Table 3
Studies investigating the association of viral and bacterial pathogens with AD and cognitive function (cohort studies).

Reference	Pathogens		Specimen type	Sample size	Diagnostic method	Increased infection burden
	Bacteria	Viruses				
Renvoize et al., 1987 [29]	*Chlamydia Group B, Coxiella burnetii, Measles, Mycoplasma pneumoniae*	HSV-1, CMV, Adenovirus, Influenza A and B	Serum	AD: 33 CTRL: 28	Complement fixation test	none
Strandberg et al., 2003 [30]	*Chlamydia pneumoniae, Mycoplasma pneumoniae*	HSV-1, HSV-2, CMV	Serum	Cohort study	ELISA	Viruses
Strandberg et al., 2005 [31]	*Chlamydia pneumoniae, Helicobacter pylori, Mycoplasma pneumoniae*	HSV-1, HSV-2, CMV	Serum	Cohort study	ELISA	Viruses
Katan et al., 2013 [26]	*Chlamydia pneumoniae, Helicobacter pylori*	HSV-1, HSV-2, CMV	Serum	Cohort study	ELISA	Bacteria & viruses
Bu et al., 2015a [27]	*Borrelia burgdorferi, Chlamydia pneumoniae, Helicobacter pylori*	HSV-1, CMV	Serum	AD: 128 CTRL: 135	ELISA	Bacteria & viruses
Wright et al., 2015 [28]	*Chlamydia pneumoniae, Helicobacter pylori*	HSV-1, HSV-2, CMV	Serum	Cohort study	ELISA	Bacteria & viruses
This study	*Borrelia burgdorferi, Chlamydia pneumoniae, Helicobacter pylori, Porphyromonas gingivalis, Treponema* spp.	CMV, EBV, HSV-1, HHV-6, HHV-7	Serum CSF	AD: 50 CTRL: 53	PCR	Bacteria & viruses

(Herrera-Landero et al., 2019; Senejani et al., 2022), our results do not necessarily refute the role of this bacterium in AD pathology. On the other hand, a direct role of CMV in the causation of AD is thought to be unlikely (Itzhaki and Klapper, 2014). Our results further confirmed a significantly higher prevalence of cases with multiple (two and more) infections in AD patients compared to controls (AD: 24 %; CTRL: 7.5 %). Miklossy previously suggested that senile plaque found in the brain is a biofilm assuring the survival of various pathogens, including *Treponema* spp. (Miklossy, 2016). While the prevalence of AD patients with no infection, single infection, and multiple infections is comparable, there is a significant difference in the prevalence of controls with and without infection. In addition, a significant difference between AD patients and controls tested negative for all studied pathogens (AD: 34 %; CTRL: 67.9 %) strongly supports a positive correlation between infectious burden and AD. It is important to note that the limitation of the present study is the fact that the samples could not be obtained for the age-matched subjects. This can be challenging, particularly in regards to the samples of brain tissue. Further investigation is needed to confirm the impact of microbial infection on AD using the samples from age-matched AD patients and controls.

5. Conclusions

To the best of our knowledge, we report the most comprehensive analysis in terms of detection method, pathogen range, and specimen types conducted thus far. Our results demonstrate that both bacteria and viruses are significantly more prevalent in AD patients than in controls. Moreover, our study provides additional evidence for the association between *Treponema* spp. and AD. Our research illustrates that paralleled analysis of multiple sample specimens provides complementary information and is advisable for future studies.

CRediT authorship contribution statement

MN – writing of the manuscript, data analysis, interpretation; TB – testing of samples and data analysis; DV – development of assays, data collection; AS – data analysis; MB – development of assays, data analysis, interpretation, supervision; MH – development of assays, supervision; BL – data collection; KS – a collection of clinical samples, interpretation; MV – a collection of clinical samples, interpretation; JH – a collection of clinical samples, interpretation; JL – a collection of clinical samples, interpretation; IK – statistical analysis; MS – statistical analysis, interpretation; RM – a collection of clinical samples, interpretation; RJ – a collection of clinical samples, interpretation; MM – supervision, funding; JD – experimental design, data interpretation, funding; All co-authors contributed to the revisions of the manuscript.

Declaration of competing interest

TB, MB and MH works in the company BioVendor. All other authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157114.

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