High Throughput Sequencing Detect Gingivitis and Periodontal Oral Bacteria in Alzheimer's Disease Autopsy Brains

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ABSTRACT

The sporadic form of Alzheimer's disease (AD) is the most common form of dementia characterized microscopically by the presence of amyloid-beta (Aβ) plaques and tau-neurofibrillary tangles with clinical presentation of cognitive deficit. Its etiology remains obscure. The cited literature from epidemiological studies suggests the presence of gingivitis and periodontitis almost double the risk for AD over a 10-year period. This feasibility study used 16S rDNA high throughput sequencing to evaluate the bacterial components of the oral microbiome in snap frozen human AD and non-AD control brains. Specimens (n=20), 10 from AD brains and 10 from non-AD age-matched brains were subjected to molecular barcoding "blindly" with high throughput sequencing. Initial PCR treatment, using 14 different primer sets separately and in combination, identified 4 (40%) positive samples in the AD-group and 6 (60%) positive samples in the non-AD-group with bacterial species associated with the oral and the gastrointestinal tract. Brain cell lysates were validated for the presence of bacterial peptidoglycan and showed 8 out of 10 brains to be positive for this universal bacterial protein in both the AD and non-AD groups. Actinomycetales and Prevotella (bacterial marker for gingivitis) and Treponema and Veillonella, (periodontitis) were present exclusively in the AD group. This study confirmed Actinomycetales and Bacteroidales (Treponema and Veillonella species) were exclusively isolated from AD brain tissue, and supports other epidemiological which demonstrate gingivitis and periodontal disease to be associated with AD.

KEYWORDS: Alzheimer's Disease; Human Brains; High Throughput Sequencing; 16S rDNA; Bacteria; Oral; Intestinal.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease and represents the most common form of dementia. Its prevalence is still rising worldwide [1]. Atrophy and neuronal death in the frontotemporal region including the hippocampus, containing amyloid-beta plaques and neurofibrillary tangles, together with a clinical history of dementia confirm presence of AD. The

clear majority suffers from the sporadic form of AD (95% of the cases) [2] in which interplay between both susceptibility genes and environmental factors is implicated [3]. One of the greatest risk factors for developing AD is advancing age, which also encompasses an aging immune system that has the potential to facilitate dysbiosis of host microbiomes.

The apolipoprotein APOE ε4 polymorphic isoform is common to all forms of AD [4]. Patients carrying the ApoE4 gene suffer from hyperlipidaemia and this predisposes them to atherosclerosis [5], bacterial infections [6,7] and the ensuing inflammation [7,8]. The ApoE4 susceptibility gene acts in combination with environmental risk factors to cause pathology. These concepts have given rise to the importance of identifying modifiable risk factors together with management of these risk factors in order to either delay or prevent the onset of dementia. For example, dysbiotic oral and gastrointestinal tract host microbiome(s) represent a potential environmental risk factor. Pathogens may spread from their primary niche to the brain, encouraging AD development [2, 9,10]. In addition, epidemiological studies identified a 10 year exposure to both gingivitis and periodontal disease promoted AD pathogenesis [11,12]. Furthermore, a prospective laboratory-based study demonstrated the directionality and the timeline of periodontal disease being a risk for AD by showing circulating antibodies to *Fusobacterium nucleatum* and *Prevotella intermedia* (oral bacteria) to be associated to a cognitive deficit 10 years later [13]. Notably S, et al. [14] showed tooth loss due to periodontal disease can almost double the risk for AD onset.

The evidence supporting that inheritance of the ApoE4 allele being an inflammatory phenotype is demonstrated experimentally via an increased secretion of inflammatory cytokines [8] and an excessively activated complement cascade [15,16]. Inflammatory mediators increase the permeability of the blood-brain barrier (BBB) to circulating pathogens, serum proteins and cytokines in the systemic circulation, providing an opportunity for the influx of dysbiotic bacteria into the brain [8,17-19]. This is illustrated by the susceptibility of the ApoE4 carriers to *Chlamydia pneumoniae* and herpes simplex virus (HSV) type 1 infections in AD brains [20-22]. Furthermore, those individuals having inherited the ApoE4 polymorphic gene [23] demonstrate Chlamydia-associated infections are able to reach the brain via infected monocytes following increased BBB permeability [24]. For a more focused review on this subject see ref [25].

Although a polymicrobial component is an essential contributor to periodontal pathogenesis, this does not completely exclude other non-microbial related factor(s) such as lifestyle behaviours (poor oral hygiene, smoking, excessive drinking, stress) and host's immune responses. A variety of microorganisms including *C. pneumoniae* [23], HSV type 1 [26] and periodontal pathogens, particularly *Porphyromonas gingivalis* (previously Bacteroides) and Treponema species have been detected in AD brains [27-30], but their relevance to the pathogenesis of this neurodegenerative disease, is debated. However, due to the recent in vivo findings of oral infections of rodents with *P. gingivalis* [17], and its association with Actinobacteria in AD brains argues at least some relevance for the pathogenesis of AD in the context of bacterial infections.

Currently there is only one published report that analysed the bacterial component of the host's microbiome in frozen and fixed post-mortem tissue from AD and control cases [31]. This initial study [31] used the 16S rDNA approach for its sensitivity and suitability to analyze bacterial DNA in frozen and fixed brain tissue.

The current study used high throughput sequencing and molecular barcoding to investigate the presence of bacterial genera in the same cohort of AD brain tissues that were examined in a previous study using biochemical and immunofluorescence methodology that detected lipopolysaccharide (LPS) as a signature of *P. gingivalis* in AD but not control cases [9].

MATERIALS AND METHODS

Bacterial DNA evaluation

Brain tissue samples: The human brain tissue used in this study was obtained from the Brains for Dementia Research (BDR) network and was kindly provided by the Newcastle Brain Tissue Resource in 2010 for a PhD project [9,32]. A new request was approved by the BDR for re-use of surplus human brain tissue samples for this study. All research procedures met approval of our academic institute (University of Central Lancashire, Ref. No. 343) and the ethical guidelines, including adherence to the legal requirements of the study in the UK. The study also received regional approval from the Norwegian REC (Regional Committee for Medical and Health Research Ethics, Southeast, Ref. No. 20152386).

The specimens were obtained from neuropathologically confirmed AD (n=10) patients comprising of eight females and two male donors with average age (79 and 83 years) respectively **(Table 1)**. Where possible, age-matched non-AD

control (n=10) brains, seven females and three males with average age (84 and 73 years respectively) were used. Samples of frozen human brain tissue were taken from an area of the brain adjacent to the lateral ventricle of the parietal lobe. On receipt, all specimens were allocated a code number and thereafter all data recorded from those cases were identified by that code. The cases are identified here as AD and non-AD controls for clarity of reporting. Related information (age, gender, cause of death, APOE allelic form, post-mortem and storage interval, number of bacteria detected by high throughput sequencing compared with the laboratory PCR methodology) for each anonymized case is given in **Table 1**.

The remaining tissue samples (approximating to 50 mg of tissue for each brain) were dissected, and genomic DNA isolation was performed in a regularly serviced PCR hood as expected per forensic DNA testing procedures. Brain specimens were thawed out in a PCR hood, and the remaining snap-frozen unfixed tissue was subjected to genomic DNA isolation at

Table 1: The age, post-mortem interval/cause of death, storage interval, and cases in which bacterial DNA detected in the cases analysed.

Case	Age and sex	Post-mortem interval (hours)	Cause of death and APOE allelic form (in bold text)	Storage interval in years	Bacterial numbers detected by high throughput sequencing	Bacterial DNA detected by lab PCR
AD 1	78/F	12	Unknown/**unknown**	21	0	Yes
AD 2	77/F	8	Unknown/**unknown**	19	19421	Yes
AD 3*	84/F	8	1a heart failure due to 1b senile dementia. **3/4**	19	1266	No
AD 4	84/F	8	Likely bronchopneumonia. **3/3**	19	0	No
AD 5*	85/F	9	Unknown. **3/3**	18	0	No
AD 6	83/M	9	Unknown. **4/4**	17	0	No
AD 7	80/F	4	Pulmonary embolism. **4/4**	17	0	Yes
AD 8*	83/F	10	Unknown/**unknown**	16	2573	Yes
AD 9	63/F	11	Bronchopneumonia. **3/4**	9	0	Yes
AD 10*	83/M	12	Unknown. **2/2**	5	5374	Yes
Non-AD 1	69/F	16	Gastric cancer. **3/3**	9	503	Yes
Non-AD 2	72/M	17	Esophageal adenocarcinoma. **3/4**	9	0	Yes
Non-AD 3	103/F	21	Pneumonia. **3/3**	9	40045	No
Non-AD 4	78/F	23	Metastatic esophageal carcinoma with broncho-esophageal fistula. **2/3**	8	2726	No
Non-AD 5	89/F	24	Heart failure 1a heart failure 1b mixed aortic valve. **3/3**	8	0	No
Non-AD 6	67/M	43	Metastatic laryngeal carcinoma (non-occupational)/**unknown**	4	0	No
Non-AD 7	81/M	34	1a pneumonia 1b: infective endocarditis. **2/3**	3	1670	No
Non-AD 8	88/F	34	1a aspiration pneumonia, 1b RT total anterior circulation stroke. **3/3**	4	7795	No
Non-AD 9	78/F	22	Metastatic cancer, primary origin unknown (probably ovarian). **2/3**	3	0	No
Non-AD 10	89/F	22	Ischemic bowel and multi-organ failure. **3/3**	3	3328	No
Total bacteria after all filtration = 84.701; * Cases positive for *P. gingivalis* LPS [9]						

the University of Central Lancashire as described below. Each specimen was placed into a sterile labelled Eppendorf® tube containing 180 µl enzymatic buffer (20 mM Tris-Cl, pH 8.0, 2 mM sodium EDTA, and 1.2% triton x-100) to which lysozyme (Sigma-Aldrich Poole, Dorset, UK) was added (20 mg/ml). All lids were closed and then covered with Parafilm® M (Sigma-Aldrich). Following an overnight incubation at 37˚C with shaking, fresh 180 µl of buffer with 20 µl proteinase K (Sigma-Aldrich) were added to each tube and the content was mixed and re-incubated overnight at 56˚C as before. Next day, the tubes from the incubator were removed and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) from Sigma-Aldrich was added to the digested sample and vortex-mixed. The tubes were centrifuged at room temperature for 5 min at 21,000 RCF. The upper aqueous phase was collected into a pre-labelled sterile, Eppendorf® tube. A cocktail (glycogen, Sigma-Aldrich, 20 µg/µl, 7.5 M ammonium acetate (Sigma-Aldrich) at pH 5.2 (0.5 × volume of sample) and 100% ethanol, 2.5 × volume of sample) were added, reagent by reagent, into the aqueous phase. The tubes were placed at -20˚C overnight to precipitate DNA. Following precipitation, the tubes containing each sample were centrifuged at 4˚C for 30 min at 21,000 RCF to pellet the genomic DNA. The pellets were washed three times in 150 µl 70% ice cool ethanol and centrifuged twice at 4˚C for 2 min at 21,000 RCF. The isolated genomic DNA (in ethanol) was sent to the University of Oslo, Oslo, Norway via an overnight courier service. All specimens were coded to ensure the experimenter remained completely unaware of which cases corresponded to AD and non-AD control brains.

PCR and high throughput sequencing

At the University of Oslo, DNA samples (n=20) were suspended in 100 µl Qiagen buffer (Qiagen Instruments AG, Stockach, Germany) and subjected to PCR using 14 different primer sets (Eurofins Genomics, Ebersberg, Germany) **(Table 2)**. Combinations of forward and reverse primers targeting the V4 region were used: 502F/701R, 503F/701R, 501F/702R, 503F/702R, 504F/702R, 507F/702F, 508F/702R, 503F/703R, 504R/703R, and 507F/703R [33]. The v3F. forsyth primer was combined with 701R-708R. PCR reactions were performed (in triplicate) for each sample. Each 20 µl volume of PCR reaction contained the following (1 µl of 10 pool/µl forward and reverse primers, 2 µl of template DNA and 16 µl AccuPrime™ Pfx SuperMix mixture (Thermo Fisher Scientific, Wilmington, DE) consisting of 22 U/ml Thermococcus species KOD thermostable polymerase complexed with anti-KOD antibodies (Invitrogen, Carlsbad, CA), 66 mM Tris-SO4 (pH 8.4), 30.8 mM (NH4)2SO4, 11 mM KCl, 1.1 mM MgSO4, 330 µM dNTPs, AccuPrime™ proteins, and stabilizers purchased from Invitrogen). Prior to PCR, pipettes, tips and Eppendorf® tubes were decontaminated under UV irradiation for 30 min in the PCR assembling hood. PCR amplifications were performed on a GeneAmp PCR System 2700 instrument (Applied Biosystems) using sterile reagents with initial denaturation at 95˚C for 5 min followed by 30 cycles of denaturation at 95˚C for 20 sec, annealing at 55˚C for 30 sec, extension at 72˚C for 60 sec, with a final extension at 72˚C for 10 min. The product was stored at 4˚C. Replicate amplicons were pooled and visualized on 1.0 % Seakam agarose gels using 10 µl GelRed Nucleic acid binding dye (Biotium Inc., Fremont, CA) in 1 × TBE buffer. The amplicons were cleaned using a SequalPrepTM Normalization Plate (96) kit (Invitrogen) according to the manufacturer's instructions. Molecular identifier (MID) tags, 10-mer, were used as sample identifiers **(Table 2)**. All pooled PCR products were purified using the Agencourt AMPure PCR purification system (Beckman Coulter, Brea, CA). The recovered DNA quantity was recorded (Nanodrop 3300 Flurospectrometer, Thermo Fisher Scientific).

High throughput sequencing was performed using the Illumina MiSeq 500-cycle v2 (2 × 250 bp) kit (Illumina, San Diego, CA), which resulted in 15 GB, 25 M SE/50M PE raw reads. Sequencing was performed at the Department of Biochemistry, DNA Sequencing Facility, University of Cambridge, UK (http://www.bioc.cam.ac.uk/dnasequencing). Processing of the sequencing data and taxonomy assignment were accomplished with an algorithm modified and based on that described by Siddiqui et al., [34]. To maximize the assignment rate, raw reads were used directly without quality filtering. Reads were first assigned with sample IDs based on the MID sequences and then BLASTN-searched against a combined set of 16S rRNA reference sequences consisting of the HOMDEXTGG set published by Siddiqui et al., [34] and the NCBI 16S rRNA reference sequence set (ftp://ftp.ncbi.nim.nih.gov/blast/db/16SMicrobial.tar.gz). These combined, well curated and near full-length reference sequences represented 1,151 oral and 12,013 non-oral microbial species.

Statistical analyses

To analyze any significant differences between the microbiota of AD and control brains a statistical method introduced in Metastats (www.metastats.cbcb.umd.edu) was used. This method employs a false discovery rate to improve specificity in high complexity environments, and in addition handles

sparsely sampled features using Fisher's exact test [35]. The Shapiro-Wilk test was used for assessing differences in the post-mortem interval and the non-parametric Mann-Whitney U test for independent samples with IMB SPSS statistical package version 23. A p-value of ≦ 0.05 was considered significant.

Table 2: PCR primers used in this study.

No.	Oligo name	Sequence
1	V4.SA501	5′-AATGATACGGCGACCACCGAGATCTACACATCGTACGTATG-GTAATTGTGTGCCAGCMGCCGCGGTAA -3′
2	V4.SA502	5′-AATGATACGGCGACCACCGAGATCTACACACTATCTGTATG-GTAATTGTGTGCCAGCMGCCGCGGTAA -3′
	V4.SA503	5′-AATGATACGGCGACCACCGAGATCTACACTAGCGAGTTAT-GGTAATTGTGTGCCAGCMGCCGCGGTAA-3′
3	V4.SA504	5′-AATGATACGGCGACCACCGAGATCTACACCTGCGTGTTATG-GTAATTGTGTGCCAGCMGCCGCGGTAA-3′
4	V4.SA507	5′-AATGATACGGCGACCACCGAGATCTACACGGATATCTTATG-GTAATTGTGTGCCAGCMGCCGCGGTAA -3′
5	V4.SA508	5′-AATGATACGGCGACCACCGAGATCTACACGACACCGTTAT-GGTAATTGTGTGCCAGCMGCCGCGGTAA -3′
6	V4.SA701	5′-CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGT-CAGCCGGACTACHVGGGTWTCTAAT -3′
7	V4.SA702	5′-CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGT-CAGCCGGACTACHVGGGTWTCTAAT-3′
8	V4.SA703	5′-CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGT-CAGCCGGACTACHVGGGTWTCTAAT-3′
9	V4.SA704	5′-CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGT-CAGCCGGACTACHVGGGTWTCTAAT-3′
10	V4.SA705	5′-CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGT-CAGCCGGACTACHVGGGTW TCTAAT-3′
11	V4.SA706	5′-CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAG-wTCAGCCGGACTACHVGGGTWTCTAAT-3′
12	V4.SA707	5′-CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGT-CAGCCGGACTACHVGGGTWTCTAAT-3′
13	V4.SA708	5′-CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAGT-CAGCCGG ACTACHVGGGTWTCTAAT-3′
14	V3F.forsyth	5′-AATGATACGGCGACCACCGAGATCTACACTATGGTA-ATTGTCCTACGGGAGGCAGCAG -3′

Bacterial peptidoglycan protein validation

Tissue lysates of all AD and non-AD brains: At the University of Central Lancashire, UK, tissue lysates were prepared from all human brains as previously reported [9] and were used for dot blots with the anti-bacterial peptidoglycan antibody (MAB995) for overall presence of bacteria in the specimens.

Dot blot: To confirm the presence of bacterial peptidoglycan, dot blots were performed by transferring 30 µg of total protein onto a polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore, UK), which had been previously permeabilized with methanol and hydrated in 1 × transfer buffer (diluted from 10 × stock transfer buffer: 144 g glycine, 30 g Tris base/L of distilled water, pH 8.3) in distilled water containing 10 % methanol. The membrane was subsequently blocked for 30 min at room temperature in 5% w/v skimmed milk/PBS then incubated overnight at 4°C with the anti-bacterial peptidoglycan antibody (MAB 995 from Millipore, UK) diluted 1/500 in 5% w/v skimmed milk/PBS. Following 3 × 15 min washings in PBS containing 0.2% tween 20, the membrane was incubated in horse radish peroxidase (HRP)-conjugated goat anti-mouse Ig secondary antibody (Chemicon, Millipore, UK) diluted 1/10,000 in 5 % w/v skimmed milk/PBS for 2 hrs at room temperature. Following further washes in PBS/tween 20, (as above, 3 × 15 min each) spots were detected using the enhanced chemiluminescence detection reagent. Specific protein signal from the membranes was visualized using a ChemicDoc® (Bio-Rad, UK) and images captured with Image Lab® Software Version 3.0.1.

RESULTS

PCR and high throughput sequencing of brain samples

Ten out of 20 samples gave PCR products. The remaining 10 samples failed even after using a set of 14 primers in different combinations. Breaking the code showed that six (60%) of the positive samples were from non-AD participants, while four (40%) belonged to AD patients. A wide spectrum of bacteria was detected in samples from both groups, containing both oral and gastrointestinal tract (GI) microbiome species. The post-mortem delay for the AD specimens on average was 9 h. However, the storage interval from donation of brains to BDR and isolating DNA, for this study, on average, overall was 16 years. For the cases that were significantly associated with the presence of bacteria in AD, the average storage interval was 15 years **(Table 1)**. The AD brain specimens without presence of bacteria were of slightly longer storage interval (17 years on average). The control, non-AD cases, on average had a longer post-mortem delay interval of 26 h compared with the AD cases with bacteria present. The storage interval with and without bacterial counts for the control, non-AD cases, on average, was 6 years. Whilst the cause of death for the AD cases with bacteria were unknown for 3 out of 4, one case resulted from heart failure **(Table 1)**. For the control group, the cause of death was clearly defined **(Table 1)** whereby 3 out of 6 cases with bacterial counts had suffered from pneumonia, stroke and bacterial sepsis (multiple organ failure). Therefore, a significant difference between the AD and non-AD groups lies in the post-mortem interval and storage interval.

The distribution of bacterial taxa was sorted into two groups comprising of AD and non-AD (not shown). In the AD group

the phylum Firmicutes was considerably higher, and the Proteobacteria phylum was much lower than in the non-AD group. The distribution of bacterial families was also determined and the dominating family in the AD group was Pseudomonadaceae. **Figure 1** shows the relative amounts of a great number of genera discovered in both groups (see **Table 1** for actual numbers of bacteria). It was noteworthy that members of the Actinomycetales order and Prevotella species (typically of *Gingivitis*) and Treponema and Veillonella species (periodontitis) were present in the AD group, but not in the non-AD group. Conversely, the genus Fusobacterium was seen in the non-AD group, but not in the AD group.

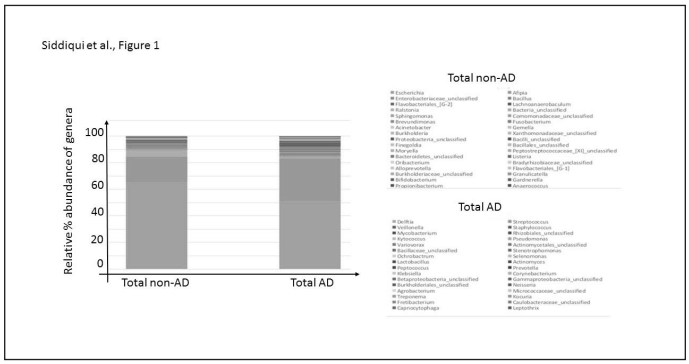

Figure 1: Bacterial reads compiled for the number of genera detected in non-AD (n=10) and AD (n=10) brains. The Y-axis represents relative percentage (%) abundance of bacterial genera.

Statistics for post-mortem delay and storage interval

The Shapiro-Wilk T-test for independent samples showed that the post-mortem interval was normally distributed, but that storage interval was not. Therefore, the non-parametric Mann-Whitney U test was used for independent samples for storage interval. In both cases the difference was highly significant (p-value=0001). Post-mortem interval mean: Shapiro-Wilk, T-test (independent samples) for AD=9 hrs and for the non-AD=26 hrs (p-value=0.0001). For storage interval mean: Mann-Whitney U test (independent samples) for AD=16 years and non-AD 6 years (p-value=0.0001).

Bacterial peptidoglycan dot blot for all brains

Dot blot analysis from tissue lysates from all the brains tested, confirmed the presence of the bacterial peptidoglycan protein. The positive control bacterial cell lysate from *P. gingivalis* (*P.g.)* was positive, and the sterile medium (Med) containing proteins used as a negative control remained negative for both groups of dot blots. Whilst non-AD brain lysates from case 2 and case 4 were negative, the non-AD (cases 1, 3, 5, 6, 7, 8, 9, 10) demonstrated unequivocal density around the specimen spots albeit with weaker density around each of the specimen spots. The AD brain lysates from the AD case 1 and AD case 10 brain lysates were negative whilst only a weaker spot appeared in AD brain lysate case 9 compared with AD (cases 2, 3, 4, 5, 6, 7, 8). The AD cases 6 and 7 brain lysates demonstrated a significantly greater density of the specimen dot spots **(Figure 2)**.

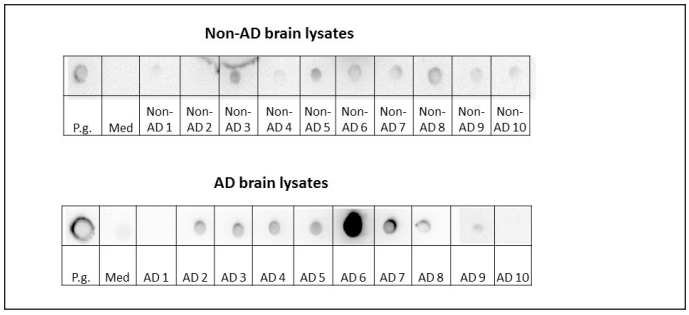

Figure 2: Dot blot using lysates from non-AD (n=10) and AD (n=10) brains, bottled with mouse anti-bacterial peptidoglycan (MAB 995). The first dot in both blots represents lysate from *P. gingivalis* (P.g.) as a positive control for Gram-negative bacteria and the med refers to sterile medium used to culture (P.g.) as a source of irrelevant protein as negative control. Non-AD 1-10 are control brains showing some bacterial peptidoglycan. The AD 1-10 brain lysates when dot blotted demonstrated a significantly greater density in AD 6 suggesting a Gram positive bacterial contribution, whilst AD 3, 5, 8 and 10 that were positive for *P. gingivalis* LPS [28] were weak in their dot blot density.

DISCUSSION

Investigating microbial infections in the context of AD brains have a negative connotation because of the potential for cross-contamination of the brain tissue obtained at post-mortem with organisms from bowel or systemic tissue. Even if compelling evidence indicates a specific pathogen to contribute to the pathogenesis of AD, it is unlikely that a single "infectious" agent is the exclusive cause of this neurodegenerative disease.

It is noteworthy that individuals with the *APOE ε4* gene are vulnerable to greater amounts of Aβ, oxidative stress, lysosomal leakage, infections and pathogen/Aβ driven inflammation. These host stressors appear to have little effect on the survival of Actinomycetales, perhaps because of their association with multispecies of bacteria, which together (as a biofilm) tolerate adverse conditions [36].

With regards to post-mortem delay, this study demonstrated a significant difference in post-mortem delay in controls (26 hrs) compared with AD cases (9 hrs). However, the storage interval at -80°C was much shorter than for control cases (6 years) compared with AD cases (16 years).

Within the Cohort, age range at death, and gender were similar, however, we cannot rule out whether or not the duration of tissue storage (16 years) had added to potential

cross-contaminations in the AD cases, despite our results showing a minimal effect. Thus, the issue is more likely to be related to post-mortem interval and DNA degradation rather than cross-contamination.

Appreciation of the use of high throughput sequencing methodology is important in view of its likely future application in "the diagnostic standard worldwide". It enables earlier discovery of novel infectious agents that may be involved in the development of complex diseases with unknown causes, which are currently difficult to detect using conventional procedures. In addition, the DNA from stored tissue can be examined retrospectively if the problem associated with degradation of DNA can be overcome.

In this study, electrophoresis of the PCR products demonstrated ten brain tissue specimens to show positive amplicons, while the remaining ten were negative. Six of the 10 positive PCR specimens belonged to non-AD participants, while the other four were from AD brains. This outcome does not necessarily mean that individual brains were free of bacteria, because DNA is known, under post-mortem conditions, to degrade rapidly. From this investigation, it appears that a time scale of post-mortem delay (9-26 hrs), and the storage interval at -80°C (16-6 years) would require further refinements.

However, using 16S rDNA high throughput sequencing, this study detected a wide range of different bacterial taxa belonging to the phylogenetic groups Bacteroidetes (Treponema, Prevotella) and Actinomycetes, exclusively in tissue from four AD (Cases 2, 3, 8, 10) **(Table 1)**. Sequencing analysis confirmed Actinomycetes exclusively in AD tissue compared with normal brains [31], and *P. gingivalis* LPS was identified in AD (Cases 3, 5, 8 and 10). Surprisingly AD case 6 (which demonstrated the most prominent dot blot signal) did not reveal bacterial DNA using the 16S rDNA high throughput sequencing or [32] using PCR methodology, suggesting that bacterial LPS may degrade more slowly than DNA.

Dot blot analysis for the bacterial peptidoglycan demonstrated bacteria to be present in at least eight brains in both (AD and non-AD) groups without significant difference. *P. gingivalis* LPS and the anti-peptidoglycan antibody did not recognize this bacterial outer membrane component, however, AD case 10 **(Table 1)** but, not case 1, provided evidence for bacterial content following PCR [9,32]. It is possible that *P. gingivalis* LPS from the outer membrane vesicles may have bound to other species of bacteria, particularly Actinomycetes; as demonstrated in AD cases 3 and 10 **(Table1)** [9,37]. In support of this finding, Actinobacteria are commonly found in the oral cavity and in the dysbiotic gastrointestinal microbiota of AD patients [10]. *P. gingivalis* outer membrane vesicles are known to assemble with Actinomyces viscosus cells [38]. Furthermore, the gene coding for a 40 kDa protein in the outer membrane vesicle protein of *P. gingivalis* has previously been cloned [39] and was confirmed as an important aggregation factor between *P. gingivalis* and *A. viscosus* [40]. The increased antibody levels to *F. nucleatum* and *P. intermedia* have been detected in AD patients [13] indicating the relevance of bacterial gingivitis/periodontitis and Actinobacteria in this study.

Understanding the AD microbiome is important, although this study shows *P. gingivalis* DNA was not recovered from either AD cases or from non-AD-cases. One explanation is the scarcity of *P. gingivalis* DNA in the brain samples, as this bacterium is found in low copy numbers, even in its primary oral niche (periodontal pocket), where it is a keystone bacterium of tissue destruction [41,42]. In addition, due to the poor quality of DNA extracted from end-stage AD brains at post-mortem, DNA from *P. gingivalis* may not have been amplified. Although not all bacteria were affected, *P. gingivalis* may be an exception in sensitivity to PCR inhibitors and other reagents that are used during nucleic acid extraction or copuriWed components from the biological sample such as bile salts, urea, haeme, heparin, and immunoglobulin Ig [43,44]. This limitation was identified and addressed in a former study; here the same tissue specimens were analyzed with biochemical and immunofluorescence methodology, detecting LPS as a signature of *P. gingivalis* in AD but not control cases (4/10) [9]. In this study, oral and intestinal bacterial DNA were also identified in the brain tissue from both AD and non-AD subjects using DNA and high throughput sequencing methodology.

CONCLUSION

In conclusion, the high throughput sequencing methodology is likely to be integrated into medical diagnostics worldwide and therefore there is a need to test its application for stored brain tissues. Our study demonstrated its applicability is plausible. It remains unclear whether or not bacteria within the post-mortem AD brain are related to systemic bacteria associated with the causes of death in AD cases, or to changes in bacterial populations following death. This study, nevertheless, confirms the presence and identification of bacterial species associated with gingivitis and periodontal

disease in the AD brains, supporting former (prospective and retrospective) laboratory based and epidemiological studies. Furthermore, this study highlights Actinobacteria possibly binding with *P. gingivalis* LPS and this has relevance for AD pathophysiology. We do not suggest that oral bacteria (gingivitis and/or periodontitis pathogens) are responsible for all AD cases, but poor oral hygiene, which promotes bacterial related periodontal disease, may be a modifiable risk for AD in some individuals.

Conflict of Interest

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