Microbial Translocation in Patients with Parkinson's Disease in Zambia: a Case Control Study

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Abstract

BACKGROUND

Over the past few years evidence has emerged that Parkinson's disease (PD) could originate from the gastrointestinal tract. Gut leakiness in patients who are genetically susceptible to PD might be an important early component to initiation and progression of the disease, via microbial translocation mechanisms, but this has not been explored in Zambia.

OBJECTIVES

To study the association of microbial translocation in patients with Parkinson's disease (PD) in Zambia.

METHODS

We conducted a case control study at the University Teaching Hospital in Lusaka Zambia between October 2019 and March 2020. We enrolled, consecutively 22 PD patients (20 previously diagnosed and 2 newly diagnosed) presenting to the neurology clinic and compared them to 44 unmatched controls from the PD patient household and non-household individuals. We measured plasma lipopolysaccharide binding protein (LBP) levels to assess systemic exposure to gut bacterial endotoxin lipopolysaccharide (LPS) and 16S ribosomal RNA (16S rRNA) gene copy number was quantified by quantitative real-time polymerase chain reaction.

RESULTS

Using Fischer's exact test there was no significant difference in the 16S rRNA gene copy number between the cases and controls (P-value: 0.122). In both groups the levels were very low to undetectable. Lipopolysaccharide binding protein levels were similar in both groups.

CONCLUSION

This data suggests that the markers of microbial translocation, 16S rRNA gene copy number and lipopolysaccharide binding protein levels, in Zambian patients with Parkinson's disease are similar to those without Parkinson's disease.

Introduction

The presence of alpha (α)-synuclein containing Lewy bodies (the hallmark of PD) in not only the central nervous system but also in neurons of the enteric nervous system has led to the hypothesis that GastroIntestinal disease might play a role in the pathogenesis of PD (1). Gut leakiness in patients who are genetically susceptible to PD might be an important early component to initiation and progression of the disease. Several studies from high income countries have shown a correlation between Parkinson's disease and gut dysbiosis, intestinal hyperpermeability, morphological changes in the intestinal epithelial

barrier, and systemic exposure to endotoxin. However there is little to no knowledge about the role of the gut in patients of African ancestry with Parkinson's disease (2)(3)(4).

The gastrointestinal lumen is in constant exposure to gut microbiota a potential source of toxins that might induce inflammatory and oxidative pathways (5)(6). Microbial translocation (MT), currently defined as the passage of both viable and nonviable microbes and microbial products such as lipopolysaccharide (LPS) across an anatomically intact intestinal barrier could be the mechanism by which the enteric nervous system could be exposed to inflammation that would later lead to the initiation of neurodegeneration in PD (7)(8).

The role of the gut in individuals with PD of African ancestry has not, to our knowledge been studied. We set out to investigate if markers of MT might correlate with PD. We hypothesized that patients with Parkinson's disease in Zambia might have elevated markers of microbial translocation, LBP and 16S rRNA gene copy number.

Methods

This was an unmatched cross sectional case control study. Approval for the study was obtained from the University of Zambia Biomedical Research Ethics Committee (006-03-19, 25th April, 2019).

Participants, both PD and non-PD were black Zambians 18 years of age and above. PD participants who met the UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria were recruited, regardless of duration of the disease (9). Controls were recruited from members of the patient's environment including spouses of PD patients who were free of neurodegenerative disease, healthy adults looking after in hospital patients, and healthy medical staff.

Written, informed consent was obtained from participants. From the study were excluded, individuals that declined consent, had parkinsonism, including history of undergoing treatment with medication that might induce parkinsonism (e.g. metoclopramide, typical and atypical antipsychotic agents), had stroke-related parkinsonism, head injury or sub-arachnoid hemorrhage, history of encephalitis, evidence of chronic liver disease, GI malignancy, diagnosis of inflammatory bowel disease and a history of GI motility drugs use.

Adults attending the neurology clinic at the University Teaching Hospital (UTH), Adult Hospital, and those admitted via the Adult Medical Emergency Unit (AMEU) and medical wards were invited to participate and recruited consecutively.

The University Teaching Hospital, Adult Hospital in Lusaka is a third level hospital and functions as the country's national referral center as well as a provincial and district hospital.

The background assumption for LBP was 26.2ng/ml based on local studies and we assumed a 20% increase in adults with PD (31.4ng/ml), with standard deviations of 6.9 in both groups (10). We had initially assumed a 90% power at 95% confidence to detect a 20% increase in LBP in PD cases and that

gave us a sample size of 38 cases for each group. However with the onset of the pandemic of covid 19, we encountered challenges meeting the target sample size as clinics were closed in order to safeguard the welfare of the participants. We thus assumed a ratio 1:2, so that for each case frequency there were two unmatched controls instead of the prior 1:1 and assumed a power of 80% with and alpha of 0.05. The resulting sample size was 22 cases with 44 controls. Stata was used for the calculations.

During recruitment a thorough clinical assessment was carried out in which, patient's demographics including age, sex, predominant PD phenotype, residency, occupation, use of tap water, use of medication for PD, family history of PD, and other comorbidities were documented using a questionnaire. Physical and neurologic examination was carried out by the investigator.

Following the clinical assessment and examination, about 5mls of blood was drawn from each enrolled participant via venipuncture and placed into EDTA bottles. The blood was centrifuged at 2700rpm for 15 minutes to extract plasma, within 24hrs and the plasma was stored at ¯80°c in the Department of Internal Medicine laboratory (TROPGAN freezer). Samples were thawed and processed using the DuoSet ELISA Human LBP Elisa kit as per standard procedure. Samples were run in duplicate concurrently with standards.

DNA Extraction Method Using Qiagen Kit

Plasma samples were equilibrated at room temperature (15–25°C) after which 20µl of QIAGEN protease enzyme was added to each 200µl of plasma sample in 1.5ml microfuge tubes. Samples which were less than 200µl were adjusted by adding appropriate volume of PBS and all samples were properly mixed after adding the enzyme. 200µl of buffer AL (containing 28µg/ml of carrier RNA) was added followed by vortexing for 15 seconds to ensure efficient lysis. The samples were incubated at 56°C for 10 minutes after which they were centrifuged to remove drops from inside the lid followed by addition of 250µl of absolute ethanol and thorough mixing for 15 seconds. The mixtures were transferred to the 2 ml QIAamp spin column and centrifuged at 8000 rpm for 1 minute, and the filtrate was discarded. The QIAamp spin columns were transferred to clean collection tubes, and each spin column was closed to avoid aerosol formation during centrifugation. Centrifugation did the cleaning of the samples was at higher speed until the QIAamp spin column was empty. Then 500 µl buffer AW1 were carefully added followed by centrifugation at 8,000 rpm for 1 minute. Spin columns were after that transferred to clean 2 ml collection tubes and 500 µl buffer AW2 was added without wetting the rim. The caps were closed, and the columns were again centrifuged at 8,000 rpm for 1 minute. Spin columns were moved to a clean 2 ml collection tubes and, 500 µl of absolute alcohol was added followed by centrifugation at 8,000 rpm for 1 minute. QIAamp columns were transferred to clean 2 ml collection tubes and centrifuged at 14,000 rpm to dry them. The columns were transferred to a clean 1.5 ml microcentrifuge tube and 200 µl buffer AVE was added and they were incubated at 56ºC for 5 minutes to increase DNA yield. DNA was eluted by centrifuging the columns at 14,000 rpm for 1 minute. The concentration and purity of DNA was spectrophotometrically determined using Nanodrop spectrophotometer 2000c (Thermo scientific, Massachusetts, USA). DNA was kept at -20ºC until needed.

Recovery efficiency of extraction method

The efficiency of the DNA extraction method was assessed by using, a known clone, 20µl of DNA from K12 American Type Culture Collection (ATCC) 92 522 with the concentration of 6.37ng/µl was added to 180µl of plasma sample from a donor considered healthy (with no bacteria in blood) followed by DNA extraction of DNA as described above. After extraction of DNA, the concentration was measured (5.3ng/µl) and the purity (260/280) was determined as 1.73 using Nanodrop spectrophotometer 2000c. The experiment was repeated three times, and the average efficiency was 5.3/6.37 or 83.2%.

Real-time quantitative PCR (q-PCR)

Real-time quantitative PCR was carried out using broad range primers for 16S rRNA. All primers were reconstituted with sterile water to final stock concentrations of 1mg/ml while the working primer concentrations were 50 nano grams. Real time quantitative polymerase chain reaction mixes were prepared quantitative PCR detection was performed using QuantiTect SYBR® Green PCR kit from Qiagen following the manufacturer's instructions. The qPCR was performed on a Rotor gene 6000 real time quantitative PCR machine (Rotor gene, North West, Australia). The reaction temperature profile consisted of an initial denaturing at 95C for 15 minutes, cycle denaturation at 95C for 15 seconds, annealing was at 60C for 30 seconds and extension at 72C for 45 seconds.

Real-time PCR

Real-time quantitative PCR was carried out using broad range primers for 16S rRNA. The Primers were reconstituted with sterile water, and the forward primer 16S F519 (5'-CAGCAGCCGCGGTAATAC-3') with the total of 249.8µg, 249.8µl of sterile water was added making a stock concentration of 1mg/ml. The reverse primer 16S R785 (5'-TGGACTACCAGGGTATCTAATCC-3') with the total of 345.2µg, 345.2µl of sterile water was added making a stock concentration of 1mg/ml. The primers were both diluted to 50ng/ml (20-fold) to get working concentration. The addition of 10µl of each primer (forward and reverse) from the stock was added to 180µl of sterile water to make 200ul.

Determination of specificity and sensitivity of the 16S real-time quantitative PCR assay

Determination of the specificity of the 16S rRNA gene primers was done using a known quantified (E.coli K12 strain). A 10-fold serial dilution of a quantified plasmid was used to establish the standard curve which was used for absolute quantification starting with 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 16S rRNA copy number per microliter (µl) and Non-Template Control (with each dilution tested in triplicate). After PCR amplification had been done, the sensitivity of PCR and standard curve was generated. The melting curves were generated and showed sharp peaks at the expected melting temperature (85.4°C ± 1.3) indicating specific detection 16s rRNA gene product.

Melting curve analysis for confirmation of amplicons

Melting curve analysis temperature of the PCR was increased, and as it was raised over a gradient, the double stranded template was denatured resulting in the SYBR green dissociate from the product and matching fall in fluorescence. At every point fluorescence and temperature intensities were measured and confirmed that targeted template was present, 50% of the double stranded template at a temperature of 83.4ºC was denatured. A melting curve analysis was carried out over a period of 20 minutes by first cooling the reaction to 60°C then heating to 99°C. Melting curve for each sample was constantly quantified during the heating process and the SYBR Green I fluorescence was plotted against temperature. The peak point in the melting curve analysis was deemed to be the melting temperature (Tm) after plotting the negative offshoot of the fluorescence aligned with temperature (-dF/dF vs. T).

Data Analysis

Data were entered on designed data collection tool then double entered onto Microsoft Office excel spread sheet using patient identification number analyzed using STATA 15.

Descriptive statistics of means and standard deviation, or frequency distribution and percentages was used to present demographic and clinical characteristics data that were continuous or categorical respectively. Where data were skewed, medians with interquartile ranges was used.

Categorical data from PD patients and controls were analyzed for association using Fisher's exact test due to the small cell values. The 16S rRNA was dichotomized and Fisher's exact test used to analyze for association between PD cases and controls. Continuous data for both PD cases and controls was analyzed for statistically significant differences with the Student's t-test when data were parametric and Mann-Whitney U test in non-normally distributed data. A level of significance less than 5%, ($p < 0.05$) was applied throughout.

Results

Of the sixty six (66) participants included in the study, twenty two (22) were PD patients who met the specified criteria and were recruited between October 2019 and March 2020. Of the forty six evaluated, forty four (44) met the criteria and were recruited as controls (with eight being family members of indexed cases). Demographic characteristics in both groups were similar and are detailed in Table 1. In both groups there were more female participants than males (male (9)/female (13) = 1:1.4 in PD and male (17)/female (27) = 1:1.5 in controls), and there was no statistical difference between the two groups based on one sided Fisher's exact test, (P-value: 0.53).

Table 1

Baseline Characteristics

	PD patients n = 22	Controls n = 44	
	n (%)	n (%)	P-value
Sex			0.533
Male	9(40.9)	17(38.6)	
Female	13(59.1)	27(61.3)	
Age (years) median (IQR)	69.5(63 – 75)	47(37 – 56)	***< 0.001***
< 40 years	2(9.1)	15(34.1)	
≥ 40 to 65 years	6(27.3)	25(56.8)	
≥ 65 years	14(63.6)	4(9.1)	
Residential areas			1.000
Urban	19(86.4)	40(90.9)	
Peri-urban	1(4.5)	1(2.3)	
Rural	2(9.1)	3(6.8)	
Education			0.497
University	1(4.5)	9(20.5)	
College	6(27.3)	12(27.3)	
Secondary	12(54.5)	16(36.4)	
Primary	2(9.1)	7(15.9)	
Uneducated	1(4.5)	0	
Family history of Parkinson's disease			0.462
Yes	3(13.6)	9(20.5)	
No	16(72.7)	33(75)	
Unknown	3(13.6)	2(4.5)	
Environmental factors			
Cigarette smoking			0.471
Never	18(81.8)	36(81.8)	
Ever	4(18.2)	6(13.6)	

Tap water			0.608
Yes	19(86.4)	34(77.3)	
No	3(13.6)	5(11.4)	
Heavy metal exposure			0.661
Yes	0	1(2.3)	
No	19(43.2)	36(81.8)	
Agricultural activity			0.287
Yes	11(50)	15(34.1)	
No	7(31.8)	3(6.4)	
Agricultural pesticide exposure			0.378
Yes	5(22.7)	7(15.9)	
No	13(59.1)	28(63.6)	
Tribe			0.452
Bowel movements per week-median (IQR)	3(2 – 7)	5.7(1 – 9)	***0.017***
Comorbidities			
Hypertension			***0.008***
Yes	15(68.2)	10(22.7)	
No	7(31.8)	22(50)	
Diabetes mellitus			0.543
Yes	4(18.2)	5(11.4)	
No	18(81.2)	27(61.4)	
HIV			0.337
Yes	5(22.7)	5(11.4)	
No	17(77.2)	29(65.9)	
MMSE median (IQR)	25.5(18 – 29)	29(26.5 – 30)	**0.002**
MMSE Severe cognitive impairment	6(30)	1(3.1)	**0.005**
BMI (kg/m^2) median (IQR)	24.4(21 – 29.3)	25(20.2 – 27.6)	0.342
UPDRS score median (IQR)	41(33 - 58)		
PD phenotype			

Tremor dominant	12(54.5)
Intermediate	2(9.1)
Akinetic-rigid	0
On levodopa therapy	15(68.2)
Deep brain stimulation	1(4.5)
Disease duration	
< 3 years	3(13.6)
3 to 5 years	13(59.1)
>5years	6(27.3)
Age at onset	
< 40 years	3(13.6)
40 to 50 years	1(4.5)
>50 years	18(81.8)

BMI = body mass index; HIV = human immunodeficiency virus; UPDRS = Unified Parkinson's Disease Rating Scale; PD = Parkinson's disease; IQR = interquartile ranges; SD = standard deviation; Tribe: 21 Zambian tribes represented.

For continuous variables median (interquartile ranges) are shown.

P-value in bold statistically significant

The LBP biomarker levels for both PD cases and controls were similar but inconclusive. A little over eighty percent (86.4%) of LBP levels for cases and over ninety percent for controls (95.5%) were over the kit reference limit (> 52460.9ng/ml). Only a minority of participants (3/22 cases and 2/44 controls) had LBP within the reference range, and the mean LBP in these was lower for cases (17661.246ng/ml vs 3615.48ng/ml in controls).

The 16S rRNA gene copy was dichotomized at 2 for both PD and the control group as sequencing was very low or undetectable. At 16S rRNA gene copy > 2 no difference was found in the presence of 16S rRNA gene copy between the two groups (11.36% in controls versus none in PD cases, p-value: 0.12). See Table 2.

Table 2. Laboratory parameters

Laboratory parameters	**PD cases n = 22**	**Controls n = 44**	
	Median (IQR)	Median (IQR)	**P-value**
WCC (x10⁹/L)	5.43(4.47–6.64)	4.9(4.36–5.53)	0.164
Hb	13.6(12.3–15)	14.2(12.3–14.9)	0.842
Urea (mmol/L)	4.15(3.49–6.15)	3.73(3.03–4.22)	0.042
Creatinine µmol/L	77.2(68.6–110.2)	78.7(69.7–95.7)	0.515
ALT (IU/L)	12.2(11.5–15.8)	17.8(13.1–28.6)	0.074
AST (IU/L)	23.4(18.55–31.6)	28.5(20.6–36.8)	0.324
ALB (g/L)	40(37.7–42.6)	40.4(47.1–43.3)	0.792
Direct marker of MT			
16S rRNA gene copy number/µL			
> 2	0	5	0.122

WCC = white cell count; Hb = haemoglobin; ALT = alanine transaminase; AST = aspartate transaminase; ALB = albumin; MT = microbial translocation

P-value in bold statistically significant

Discussion

The data suggests that Zambian patients with PD have very low to undetectable levels of 16S ribosomal RNA, comparable to controls. Unexpectedly, more amplification was noted in the controls. It was thought that controls might have states that positively correlate with 16SrRNA gene copy numbers such as hepatosplenic schistomiasis and environmental enteropathy in the local setting (11). There was no evidence of hepatosplenic schistosomiasis in both groups on clinical evaluation and so this condition was thought to be less likely. However with environmental enteropathy being a subclinical disorder whose identification requires endoscopic visualization of the intestinal architecture, and none of the participants had endoscopic evaluation, it could not be excluded as an underlying process leading to microbial translocation. It was not apparently clear why PD cases had undetectable 16SrRNA despite sharing similar environments with the controls. While there is emerging evidence of gut microbiome contributing to Parkinson's disease onset, a recent study from China showed no significant difference in 16S rRNA structure and richness in PD and non PD cases (12). The study investigated microbiota communities in the blood of 45 PD patients and their healthy spouses using 16S rRNA gene sequencing at Ruijin hospital,

in Shanghai, China. They also investigated the genera in the blood of the PD patients and the healthy controls which was not done in our study, and found an enrichment of certain genera in the PD group. There exists in the human gastrointestinal tract an extensively diverse microbial ecosystem which is responsible for maintaining physiologic functions of the host. Different groups of bacterial exist in beneficial proportions maintaining health. Digressive gut microbiota composition has been associated with several diseases and disorders. 16SrRNA sequencing is limited at identifying closely related species but is able to analyze differential abundances of different genera. Investigation of the genera was not done as it was not part of our aims.

LBP values were markedly elevated and comparable in both groups. This might suggest the presence of chronic low-grade inflammation in both groups. Low-grade chronic inflammation is associated with most non-communicable diseases such as diabetes mellitus, obesity, malignancy, musculoskeletal disorders, respiratory and neurodegenerative disorders as well as infection with gram-negative bacteria. Apart from diabetes mellitus and obesity there was no evidence of malignancy, musculoskeletal disorders, respiratory or neurodegenerative disorders other than PD. We could not assess the impact of diabetes mellitus, obesity and PD on LBP as nearly all the values were above the reference range in both groups. Only a minority from both the cases and controls had values within the reference range with an apparent difference in means, but these were too few to determine factors that might correlate with LBP. We encountered challenges acquiring LBP kits to run the tests again, due to COVID-19 pandemic restrictions. This coupled with the very low to undetectable 16S rRNA gene copy numbers prevented us from evaluating the influence of MT on PD phenotypes.

Bowel transit time per week a surrogate for gastrointestinal status was different in the two groups, being of low frequency in the PD than controls but could not be correlated to 16S rRNA because of low detection.

The study had a number of limitations. The first being the relatively small sample size which impacted our ability to appreciate the difference in markers of microbial translocation in the two groups. However, we applied the one case to two controls in the our study to gain on power when we failed to get the calculated sample size due to the covid 19 pandemic which restricted the clinic visitations where we obtained our participants. Secondly, only 16s rRNA gene copy number for markers of MT could be analyzed but the very low to undetectable levels of 16S rRNA copy numbers in both groups, limited our ability to further identify its impact on PD and PD phenotypes, and the impact of MT on PD. Other markers of MT would have provided rich clinical data and interpretation. The LBP values were markedly elevated and above the reference range in nearly all the participants limiting our ability to determine factors that might have an influence on LBP as it relates to PD and controls. Challenges in acquiring LBP kits to re- run the assays were encountered due to the COVID 19 pandemic restrictions. The third limitation may have come from selection of controls that were younger and others who were family members of indexed cases. While this may have controlled for the environment and diet, it did not control for gut microbiota changes that may come with aging. A fourth limitation resulted from our inability to

analyze alpha-synuclein a hall mark of PD in our cases. However Parkinson's disease is diagnosed clinically.

Conclusions

Our data showed low to undetectable levels of plasma 16S rRNA gene copy numbers in both PD and controls. This data is preliminary and suggests similar levels of MT in both PD and controls, but further studies are needed to provide insights into proportions of genera as it might be different between the groups.

Abbreviations

AMEU..... Admission Medical Emergency Unit

DNA ..... Deoxyribonucleic acid

EE..... Environmental Enteropathy

HSS ..... Hepatosplenic schsitosomiasis

LBP ..... Lipopolysaccharide binding protein

LPS ..... Lipopolysaccharide

PD ..... Parkinson's disease

MT ..... Microbial Translocation

rRNA ..... Ribosomal Ribonucleic acid

UTH ..... University Teaching Hospital

WCC ..... White cell count

Declarations

Consent for Publication:

Approval for publication was obtained from the National Health Research Authority (NHRA00025/03/02/2022, 3rd February 2022). All methods were conducted in accordance with the relevant guidelines and regulations.

Ethical Approval:

This was obtained from the University of Zambia Biomedical Research Ethics Committee (006-03-19, 25th April, 2019).

Consent to Participate:

Written and informed consent was obtained from all participants and all methods were conducted in accordance with the relevant guidelines and regulations.

Availability of Data and Materials:

The dataset generated and analyzed are available from the corresponding author upon reasonable request.

Competing Interests:

None

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Author Contributions:

PK, MA, LC conceived and designed the study. LC, PK, ES, analyzed the data. PK contributed reagents, materials, analysis tools. LC, ES, PK, MA wrote the paper.

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