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Case Report

A First Reportable Case of Fatal Granulomatous Amoebic Encephalitis In An Immunocompetent Nigerian Confirmed By Molecular Studies-Polymerase Chain Reaction (PCR)

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ABSTRACT

Aims: To demonstrate the DNA sequences of *Acanthamoeba castellanii* strain using molecular genomic DNA extraction and polymerase chain reactions.

Materials and Methods: Genomic DNA extraction: Four 5 µm thick sequential sections were cut from the formalin-fixed paraffin embedded tissue sample. The micro-sections were placed in a micro-centrifuge tube. Sections were de-paraffinized using 300 µl of mineral oil and incubated at 90°C for 20 minutes to dissolve the wax. The tissue was digested with 50 µg/ml proteinase K at 48°C and incubated overnight. Genomic DNA (gDNA) was extracted from the solution by adding an equal volume of chloroform/isoamyl alcohol (24:1) to the tube. The gDNA was determined using Nanodrop ND-1000 Spectrophotometer.

Polymerase chain reaction (PCR) assay: PCR assay was carried out on the extracted gDNA to amplify a target sequence of 161bp using Bioneer AccuPower® PCR PreMix in a reaction volume of 20 µl containing 1U of *Top* DNA polymerase, 250 µM dNTPs, 10 mM Tris-Hcl (pH 9.0), 30 nM Kcl, 1.5 mM $MgCl_2$, 25 pmol each of the two primers—forward—Aca16Sf1010 (5'-TTATATTGACTTGTACAGGTGCT-3') and reverse—Aca16Sr1180 (5'-CATAATGATTTGACTTCTTCTCCT-3') and the template DNA.

Results: PCR analysis/sequence analysis and alignment: The amplification of the 16S small subunit ribosomal RNA gene produced an expected band, which is 161bp in size. A nucleotide BLAST search was carried out at NCBI to ascertain what the sequence was. The forward and reverse sequences showed great similarity with the same sequence as given by *Acanthamoeba castellanii* strain sequence ID: gb|AF479520.1| 16S small subunit ribosomal RNA gene.

Conclusion: The high index of suspicion for *Acanthamoeba species* in this index case presenting histologically as granulomatous amoebic encephalitis was confirmed by molecular polymerase chain reaction as *Acanthamoeba castellanii*.

Key words: *Acanthamoeba castellanii*, Granuloma, Amoebic Encephalitis, Polymerase chain reaction, Molecular analysis, Nucleotide sequencing.

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INTRODUCTION

Studies have it that free-living amoebae causing infection of the central nervous system in humans include *Naegleria fowleri, Acanthamoeba spp, Balamuthia spp and Hartmanella spp. Naegleria fowleri* usually cause acute fulminant meningoencephalitis in immunocompetent individuals. *Acanthamoeba species* usually cause chronic fatal encephalitis in immuno-compromised individuals which is known as Granulomatous Amoebic Encephalitis. However this disease entity is extremely rare in immunocompetent patients.[1]

Case Report

A 31 year old female was referred from a primary health Centre with complaints of persistent headache for 10 days and irrational talk of one day duration. The headache was worse in the frontal region, associated with blurring of vision and non-projectile vomiting. There was no report of fever or associated seizure. There is no positive history of migraine headache, hypertension or diabetic mellitus. The irrational talk was not associated with hallucinations. There was no history of alcohol intake or use of psychoactive substances. There is a positive history of regular visitation to France prior to the onset of her illness.

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On examination, she was afebrile, had no signs of meningeal irritation or neurological deficit. Fundoscopy showed raised intracranial pressure hence lumbar puncture was not done. A cranial computed tomographic scan was normal. The retroviral screen was negative and the random blood sugar was normal.

The patient was admitted with initial clinical assessment of migraine headache to rule out encephalitis. She was placed on analgesics, 5% dextrose saline, 500 ml 6 hourly, 20% mannitol 250 ml 6 hourly, diazepam 10 mg via infusion and ceftriaxone (intravenous) 1g daily. On the second day of admission, she developed high grade fever (40°C) and became restless. This was followed by nuchal rigidity and positive Kernig's sign on the third day of admission. Patient was reviewed and the clinical diagnosis was changed to meningoencephalitis and the intravenous doses of ceftriaxone and diazepam were increased to 2g and 20 mg 12 hourly respectively. Intravenous 20% mannitol 250 ml 6 hourly was sustained. On the fourth day of hospital admission, her condition deteriorated and she died during the early hour of the same day. A clinical request for autopsy was made and this was done.

Autopsy finding: On external examination, she was cyanosed and moderately pale. The cerebrum is swollen (flattened gyri and narrow sulci) with prominent congested leptomeningeal vessels and perivascular fibrinous exudates. Similarly the cerebellum was oedematous, with congested blood vessels and perivascular fibrinous exudates. There was bilateral grooving of the cerebellar tonsils with tonsillar herniation. The heart was enlarged and weighs 400g with fibrinous exudates on the pericardium. The left ventricular wall thickness was 2.0 cm in diameter. The circumference of the mitral valve was 6.9 cm with fibrotic leaflets. The liver was enlarged and weighed 1850g. The outer and cut surfaces appear yellowish with nutmeg pattern. The right and left kidneys were enlarged and weighed 190g and 200g respectively. Cut sections revealed a distinct corticomedullary differentiation. The spleen was enlarged and weighed 200g. There were fibrinous exudates on the cut surface. The mesenteric lymph nodes were enlarged.

Histo pathological examination of the cerebral and cerebellar parenchyma, spleen, liver, colon and lymph nodes show similar pathology of non-caseating granulomas comprising of epithelioid macrophages and lymphoplasmacytic inflammatory infiltrates with multiple multinucleated giant cells. Ziehl-Neelson and PAS stain was negative.

Blood vessels filled with structures with morphologic characteristics of amoeba trophozoites without inflammatory reaction are noted. Also seen is fibrinoid panarteritis with thrombosis.

The anatomical diagnosis of chronic granulomatous meningoencephalitis was made.

MATERIALS AND METHODS

Molecular analysis: Genomic DNA extraction

Four 5-µm thick sequential sections were cut from the formalin-fixed paraffin embedded tissue and placed in a micro-centrifuge tube. The section was added 300 µl of mineral oil and was incubated at 90°C for 20 minutes to dissolve the wax. The tissue was digested with 50 µg/ml proteinase K (Roche Diagnostics, Mannheim, Germany) at 48°C and incubated overnight. Genomic DNA (gDNA) was extracted from the solution by adding an equal volume of chloroform/isoamyl alcohol (24:1) to the tube. The organic and aqueous layers were gently mixed for 5 minutes and spun at 13,000 rpm for 20 minutes. The upper aqueous layer was removed into another sterile eppendorf tube and an equal volume of 100% ethanol was added, mixed and was incubated at -20°C overnight in order to enhance DNA precipitation. The solution was spun at 13,000 rpm for 20 minutes and the pellet was washed with 70% ethanol and was spun for another 20 minutes. The supernatant was removed and the pellet was dried at room temperature. The pellet was re-suspended in 25 µl of double distilled water and the purity as well as the concentration of the gDNA was determined using Nanodrop ND-1000 Spectrophotometer (Nanodrop technologies Incorporated, USA), and was stored at 4°C until use.

Polymerase chain reaction (PCR) assay

PCR assay was carried out on the extracted gDNA to amplify a target sequence of 161bp using Bioneer AccuPower® PCR PreMix (Bioneer, Alameda, CA, USA) in a reaction volume of 20 µl containing 1 U of *Top* DNA polymerase, 250 µM of dNTPs, 10 mM of Tris-Hcl (pH 9.0), 30 nM of Kcl, 1.5 mM of $MgCl_2$, 25 pmol of the two primers—forward—Aca16Sf1010 (5'-TTATATTGACTTGTACAGGTGCT-3') and reverse—Aca16Sr1180 (5'-CATAATGATTTGACTTCTTCTCCT-3')[2] including the template DNA. All PCR amplifications were performed with Hybaid thermal cycler (Hybaid, OMN-E Thermal Cycler) and a thermal profile involving 5 min at 95°C, followed by 45 cycles each of 30 seconds at 95°C, 1 min at 52°C, followed by 1 min at 72°C and a final elongation step at 72°C for 15 minutes. The amplified product was visualized on 2% agarose gel stained with ethidium bromide at a concentration of 0.5 µg/ml. Photo documentation was performed with Gel Documentation and Analysis System (Clinx Science instruments, USA).

Nucleotide sequencing and alignment

Gel slice containing the target fragment was excised from agarose gel and purified with Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol and was selected for subsequent sequence analysis. Both the forward and reverse strands of the purified PCR products were sequenced using a dilution of the original PCR primers. Sequencing was performed on Applied Bio systems ABI 3500 × L Genetic Analyzer platform using Sanger dideoxy sequencing approach. The sequence has since been submitted to the GenBank database and was given accession number KP272136.

RESULTS

PCR analysis, sequence analysis and alignment

The amplification of the 16S small subunit ribosomal RNA gene produced an expected band, which is 161bp in size as shown in (Figure 1). A nucleotide BLAST search was carried out at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ascertain what the sequence was. The forward and reverse sequences showed great similarity with the same sequence as given by *Acanthamoeba castellanii* strain sequence ID: gb|AF479520.1|16S small subunit ribosomal RNA gene.

DISCUSSION

Involvement of free living amoeba in human disease was first recognized in 1965 after fatal cases of meningo-encephalitis were described in Australia and later in the United States. Free living amoebae are widely distributed globally though clinical cases have been reported mainly in Europe and North America, and recently in Asia. There is a lot of debate about how the disease is contacted but it is generally agreed that it is through skin ulcers, keratitis (especially among contact lens users) and by swimming in warm soil contaminated pools whether indoors or outdoors. The early symptom is sporadic headache which becomes constant as the disease progresses. From this point on, the disease runs a short course of 10-21 days if untreated. Free-living amoebae that cause infection of the central nervous system in humans include *Naegleria fowleri, Acanthamoeba spp, Balamuthia spp and Hartmanella spp*. *Naegleria fowleri* usually cause acute fulminant meningo-encephalitis in immune-competent individuals, whereas *Acanthamoeba* species usually

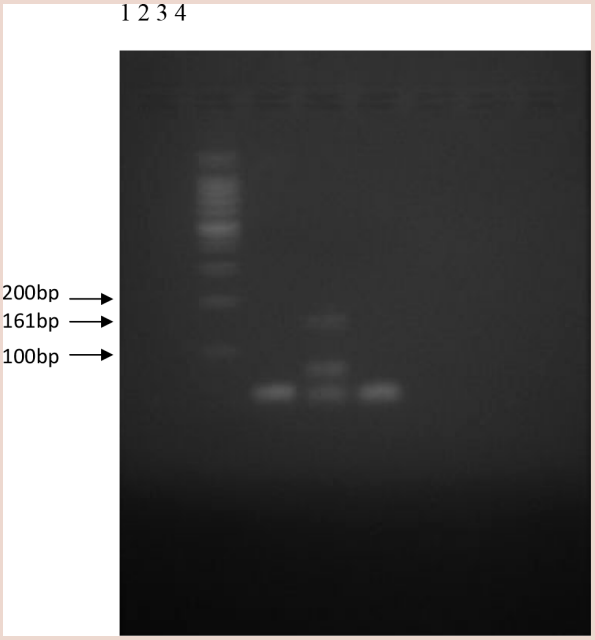

Figure 1: Agarose gel stained with ethidium bromide showing the PCR result. Lane 1: DNA size marker (100bp DNA Ladder: Promega, USA); lanes 2 and 4: negative controls (no DNA); lane 3: generic-specific amplification product from Acanthamoeba specie (size: 161bp) obtained from amplification product of genomic DNA extracted from the paraffin embedded tissue sample.

cause chronic but fatal encephalitis in immunocompromised individuals called Granulomatous Amoebic Encephalitis.[1,3,4] The organism can remain in the body without causing any symptom.

This index case was a young female who had sudden severe frontal headaches, irrational talk, bilateral papilloedema and high grade fever and positive Kernig's sign are in keeping clinical diagnosis of meningo-encephalitis. Correct diagnosis by the clinician can be a challenge without a high index of suspicion as the symptoms are non-specific. Cranial computed tomography scan may not show any abnormality as it was in this index case. Cerebrospinal fluid microscopy which would have yielded trophozoites was not done because of raised intracranial pressure.[4]

The histology however showed non-caseating nodular granulomas in the cerebrum, cerebellum, liver, spleen and mesenteric lymph nodes. An exhaustive search including special histochemical stains (Ziehl-Neelson and Per-iodic Acid Schiff) were done to rule out other possible aetiological agents and other differential diagnosis in this environment including mycobacteria and fungi infections.

In this index case examination of histological sections of the brain revealed the presence of amoebae within blood vessels and also perivascularly. Granulomas with inflammatory giant cells, extensive lymphoplasmacytic infiltrates, necrotic areas and congested blood vessels were also seen.

The current gold standard for diagnosis of granulomatous amoebic encephalitis is immunofluorescence which is however unavailable at our Centre. However molecular studies (PCR and Genetic sequencing for speciation) were done and the causative organism has been identified as *Acanthamoeba castellani*.[2-5] As the disease progresses, the cause of death is cardiorespiratory failure secondary to the compromise of the cardiorespiratory centres in the midbrain as happened in this case.

CONCLUSION

The high index of suspicion for *Acanthamoeba species* in this index case presenting histologically as granulomatous amoebic encephalitis was confirmed by molecular polymerase chain reaction as *Acanthamoeba castellanii.*

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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