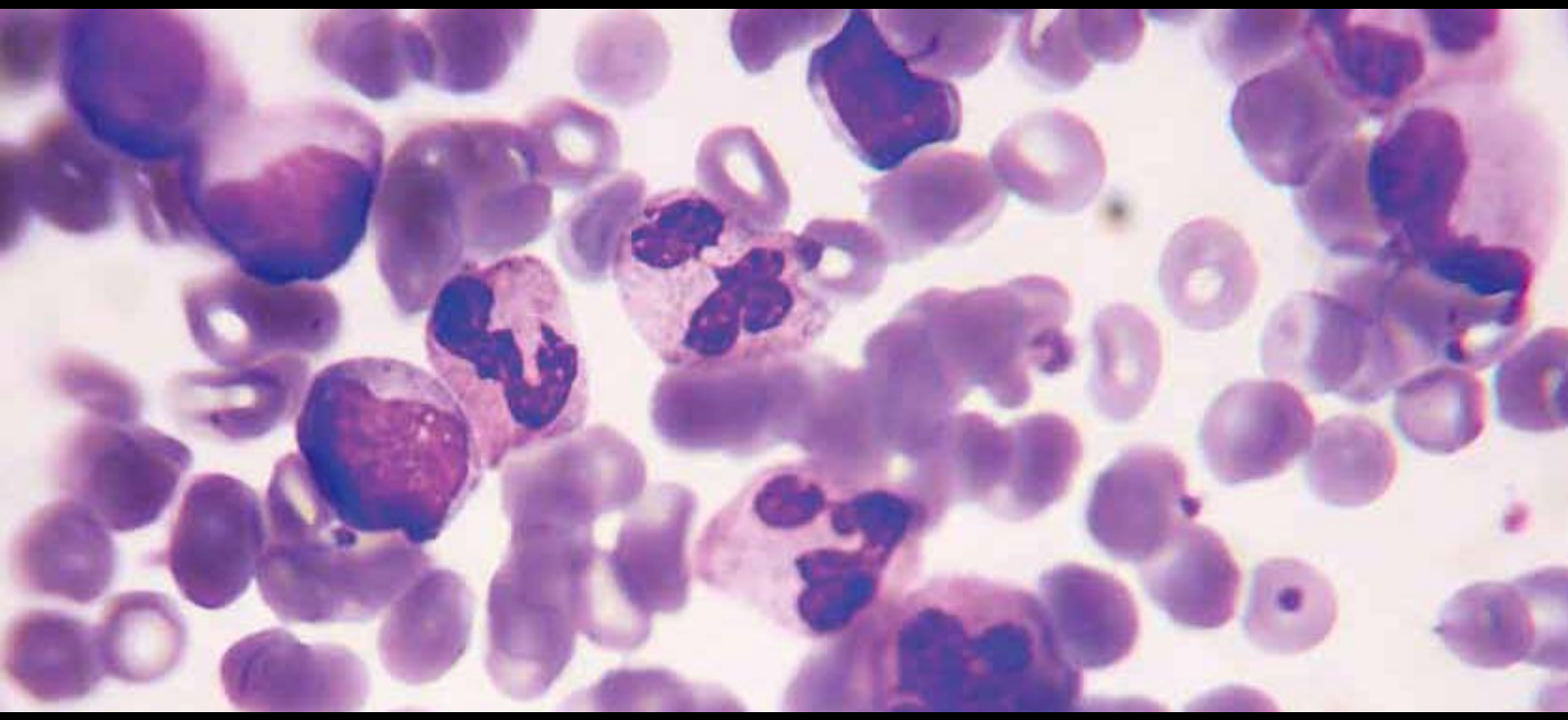
Pathology Research International

Pathology and Diagnosis of Central Nervous System Infections

Guest Editors: C. Sundaram, S. K. Shankar, Wong Kum Thong, and Carlos A. Pardo-Villamizar



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Editorial

Pathology and Diagnosis of Central Nervous System Infections

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Infections of the central nervous system (CNS) are important because of the many pathogens, the emerging and reemerging of new infections, and the heavy burden they impose on health care system. The most formidable challenge is the increasing number of people at risk of developing CNS infections due to acquired immunodeficiency syndrome (AIDS) and the recipients of hemopoietic and solid organ transplantation and other causes of immunosuppression. There have been significant developments in the last few decades in understanding the biology of disease process by the application of new technologies. Advancements in brain imaging and newer diagnostic modalities have achieved early diagnosis helping patient management with new therapies. The pathogenetic factors contributing to neurovirulence of the pathogens have been elucidated by the use of molecular biological techniques.

The infections are caused by wide variety of organisms including bacteria, parasites, fungi, and viruses. The clinical course may be acute, subacute, or chronic depending on the pathogen, location, and immune status of the host. The clinical manifestations are protean.

The normal brain is a highly complex and specialized organ. It is protected by bony encasement of skull and thick dura mater. However, this encasement limits its capacity to swell in case of inflammation. Another important defense for brain is in the form of blood brain barrier (BBB), made up of a system of tight junctions in capillaries that resist the entry of the inflammatory cells, pathogens, and macromolecules into the subarachnoid space. The brain has a rudimentary lymphatic system. The microglia and perivascular macrophages have much lower expression of major histocompatibility complex (MHC) molecules. This helps the pathogen by poor antigen presentation to body's immune system and greater survival.

The exact mechanisms to breach the BBB by certain pathogens are poorly understood, for example, rabies virus and herpes simplex virus travel within peripheral nerves to enter CNS, whereas encapsulated bacteria and fungi enter from the blood stream and possess surface components that allow them to traverse the capillary tight junctions. The natural CNS parasites (*Naegleria fowleri*) infiltrate and infect the CNS of healthy host by targeted attacking of the BBB's endothelial cells. The opportunistic CNS parasites (*Toxoplasma gondii*) infect the CNS in immunocompromised patients when the body is unable to effectively resolve inflammation of the BBB's endothelial cells, increase the permeability of the BBB, and allow large molecules and parasites to cross BBB and enter the brain.

Owing to the limited space and involvement of vital areas, CNS infections are associated with high morbidity and mortality. Rapid diagnosis and emergent interventions are necessary to improve outcomes of these patients. The laboratory diagnosis of CNS infection is essential for optimal therapy. However, it is most challenging and appropriate that the use and selection of laboratory tests requires close interaction between clinician and laboratory personnel. A timely cerebrospinal fluid (CSF) examination can give wealth of information. Apart from cell count, the CSF can be subjected to Gram's stain, fungal stains and culture of bacteria, fungi, and mycobacteria. Moreover, viral meningitis and encephalitis can be diagnosed by CSF serology, viral DNA markers, and polymerase chain reaction (PCR). Tissue diagnosis depends

upon location of the infectious focus and possibility of a biopsy from the site. Histopathology almost always gives a clue to the underlying infectious agent with the help of special and immunohistochemical stains for various types of organisms. The diagnostic yield can be further improved by the application of PCR and other molecular techniques.

Brain abscess is a focal suppurative process within the brain parenchyma, commonly caused by bacterial, fungal, and parasitic pathogens. Despite advances in diagnostic and surgical methods and advent of new antibiotics, brain abscess continues to be a serious medical problem. The predisposing factors vary in different parts of the world. Because of improvements in the treatment of ear, sinus, and orofacial infections over the last 50 years, there is decreasing incidence of brain abscess due to otogenic infections in developed countries when compared to developing countries. However the frequency of brain abscess increased in patients with AIDS and in patients using broad spectrum antibiotics, corticosteroids, or immunosuppressive agents.

V. Lakshmi et al. in a review of 352 brain abscess samples in 24 years from a developing country like India observed that otogenic infections and sinus infections by contiguous spread constitute the commonest source for brain abscess. There were only 4 patients who were immune suppressed, and two of them had mycotic abscesses. Cryptogenic brain abscesses constituted 23.3% of abscesses. *Staphylococcus aureus* was the most common isolate. The authors observed a change in the trend of the causative organisms in the later half of the study period when unusual organisms like *Burkholderia pseudomallei, Salmonella typhi, Nocardia spp, Cladosporium bantiana, Fonsecaea pedrosoi, Entamoeba histolytica, and Acanthamoeba* were isolated. There were 8 brain abscesses due to mycobacterium tuberculosis, 3 *Nocardia spp* and 5 mycotic organisms and 2 amoebic abscesses. The diagnosis of these group of brain abscess was complemented by histological studies. Tuberculous brain abscess is essentially a histological diagnosis wherein the wall of the abscess lacks granulomas, and the central necrotic material contains acid fast bacilli.

The factors for a favorable outcome for brain abscess include being male, having a Glasgow corna scale score >1.2, and being sepsis-free and having positive culture. Identification of microorganisms in the aspirated material depends on the prompt examination of smear and appropriate culture techniques. The mortality and morbidity and long-term sequelae of brain abscess are due to persistent release of proinflammatory mediators by activated microglia, astrocytes, and infiltrating inflammatory cells along with disruption of BBB. Anti-inflammatory drugs along with specific antimicrobial agents help in minimizing damage to the adjacent brain parenchyma.

Most protozoal infections except cerebral malaria are uncommon and are restricted to particular geographical regions. Because of increasing international travel, parasites that were previously limited to tropical regions pose an increasing infectious threat to populations at risk for acquiring opportunistic infection, especially people with human immunodeficiency virus (HIV) infection or individuals who have received a solid organ or bone marrow transplantation. Though CNS is an immunologically privileged site, the parasites try and gain access to the CNS due to a variety of factors which include easy access to nutrition and the ability to avoid much of the body's normal immune response. Though CNS may be one of the many systems involved, CNS involvement indicates a poor prognosis in the protozoal infections.

Though only a relatively limited number of parasites can penetrate and infect the CNS, the parasites employ a variety of techniques to evade and suppress immunity and exploit the milieu for survival. Understanding the host parasite interactions and pathogenesis helps develop efficacious treatment strategies. Detailed neuropathological studies with application of molecular biological methods helps in this direction. The awareness of endemicity and geographical distribution of parasites is necessary for proper planning of the laboratory tests. Serological tests, culture and molecular methods, are very useful in the diagnosis of parasitic infections. L. Chimelli in her paper stressed the importance of morphology of the parasites on tissue in establishing the diagnosis. She stressed the changing patterns of some protozoal infections of CNS after the institution of highly active antiretroviral therapy (HAART). The diagnosis remains a problem in many patients despite all the available tests and examination of brain at autopsy may become inevitable in making a diagnosis.

Fungal infections of CNS are being increasingly reported in the last few decades due to increase in the number of immunosuppressed individuals. A variety of fungi cause infections of CNS either an acute or chronic meningitis or space occupying lesion. Yeast fungi predominantly cause meningitis, and mycelial fungi cause mass lesions of brain. The type of pathology and clinical syndrome is determined by the morphology and size of the fungus and the host immune status. In the patients with intracranial mass lesions, direct extension from colonized paranasal sinuses or ear canal is more common than by hematogenous dissemination from lung, gastrointestinal tract (GIT), or skin. Aspergillus sp is the most common agent to cause intracerebral granuloma or abscess.

C. Sundaram and J. M. K. Murthy reviewed intracranial aspergillus granulomas and observed that most of the reported large series are from countries with temperate climate like India, Pakistan, Sudan, and Saudi Arabia. The spread is often from paranasal sinuses by direct extension and in immune competent hosts. The lesions are often extracerebral granulomas, characterized by dense fibrosis. Rare intraparenchymal granulomas were reported. The importance of histochemical stains like Gomori methenamine silver in the diagnosis is stressed. Environmental factors like temperate climate humidity favor the growth of the fungus. The aerolized spores during ploughing or construction activity are colonized in the sinuses or lungs. Dissemination to CNS occurs due to local altered immunity and mucosal invasion. The dense fibrosis does not allow effective penetration of antifungal agents thus necessitating radical surgery.

Following HIV infection, especially with HIV encephalitis, variable degree of demyelination is found in the brain, especially the subcortical white matter of the frontal and temporal lobes. This is further accentuated by coinfection with

JC virus causing progressive multifocal leucoencephalopathy, involving the white matter fiber tracts, basically infecting the oligodendroglia. S. Surendran et al. in their earlier molecular studies showed the presence of human aspartoacylase in oligodendroglia taking part in myelin synthesis. Altered levels of aspartoacylase/aminoacylase (ASPA) and abnormality in the metabolic pathway have been found to induce oxidative damage and participate in the evolution of Canavan's disease and Parkinson's disease. In a brief report in this issue, they described depletion of immune labeling for ASPA protein in the white matter in cases of HIV encephalitis. This indicates that an aberration in ASPA pathway participates in the demyelinating pathology seen in cases of HIV. More in depth study with larger autopsy sample size is needed to further validate the observations and suggest a pathogenetic role.

With transformation of the world into a global village with primitive to advanced modes of transport, old infective conditions are emerging in new places, and same diseases are manifesting with varied clinical features confounding the infectious disease specialist and the laboratories. Many of them are turning out to be zoonoses of well-recognized or newly discovered viral infections, jumping the species barrier and infecting the human beings. This is further facilitated by the economic necessity of close proximity of the domesticated animals and the human beings, afforestation leading to migration of the animals into human habitat. K. T. Wong and K. C. Ong have provided a brief review of an encephalitis, glomerulonephritis, bronchitis, and bronchiolitis caused by Hendra virus and Nipah virus originating in the Eastern World. The viral infection has spread to humans essentially by contaminated oropharyngeal secretions and urine from infected domestic animals. The intermediate host is essentially determined by the proximity to the human habitat and domestic animal rearing habit of the villages involved. The person-to-person transmission in Bangladesh and India by the henipaviruses reflects high human density in a restricted area and aerosol spread like the recent mutated influenza virus epidemic. The clinical features of Hendra virus has not been well characterized while Nipah viral infection is very well studied and recorded.

With henipavirus infection, probably viral persistence is responsible for recurrent and recrudescent encephalitis, similar to a few of the arboviral infections. The site of latency of the virus is not clear. The pathology of henipavirus is diffuse parenchymal vasculopathy, endothelial cell syncytia, and encephalitis. In Nipah virus infection CNS vasculopathy is more prominent, thus highlighting variable pathologies in henipavirus group of infections. Similarly the susceptibility of the experimental animals is also variable, modulating the spread of infection in the natural habitat. Curiously in case of Nipah viral infection, role of peripheral nerves in the viral transmission to CNS had been suggested only in pigs.

The formation of neuronal syncytia indicates the activity of fusion viral protein, a feature of paramyxoviruses. Sharing the same viral receptor on the cell membrane of both humans and animals (ephrin B_2 and ephrin B_3), especially the vascular endothelium explains the evolution of clinicopathological features and CNS involvement. With changing ecology following the expansion of human habitat and associated domesticated animals, disturbing the environment and homes of pteroid bats in developing countries, future outbreaks of henipavirus can be anticipated. This calls for evolving treatment strategies and vaccination policies.

In Asian countries rabies viral infection continues to be a public health problem with no reliable cure in sight. WHO estimates 50,000 deaths every year worldwide, nearly 60% of them occurring in India alone. Humans and canines, the common mammals infected, acquire the disease following the bite of a rabid animal. On entering the nervous system by a receptor-mediated mechanism, the virus replicates and spreads widely in the CNS with a fatal end. Clinically rabies infection manifests either as furious (encephalitis) or paralytic form, two thirds of the subjects suffering from the aggressive, hydrophobic, and furious form. Most of the studies evaluating the pathogenesis of rabies have been carried out in laboratory animals using laboratory-adopted virus strain (CVS), while the natural infection by the nonattenuated "street virus" in humans and canines is not well worked out.

In addition to cytopathic effect of the viruses, altered neurotransmitter activity resulting in deleterious CNS physiology have been incriminated as the cause for acute morbidity and mortality following infection with neurotropic viruses like rabies. Apoptosis, which is essential for the programmed cell death and embryogenesis, has been observed in a multitude of viral infections, and the number of correlations between viral pathogenesis and apoptosis continues to grow. Some of the viruses promote noninflammatory apoptotic mechanism to induce cell death and escape into the interstitium to infect another healthy cell. On the other hand, other viruses cleverly exploit the high regulated apoptotic pathway by blocking it within the cells they reside, thus evading the host surveillance mechanism and promote their survival. RNA viruses multiply rapidly to produce many virions before the host mounts effective immune surveillance to contain them. Observation of apoptosis in mouse neuroblastoma cells when infected with highly neurotropic challenge virus standard (CVS) of rabies virus leads to the impression that neuronal damage and loss in rabies is mediated by apoptosis. Subsequent workers as well, using the laboratory passaged CVS strain and animal models, have suggested that apoptotic neuronal cell loss was an early event correlating with disease severity. Though apoptosis is well recognized in animals infected with laboratory-adopted-rabies virus, whether the same mechanism is operative in natural infection by rabies virus present in nature remained a moot point.

The neuronal apoptosis following rabies infection is found to be age dependent, being evidence in suckling mice infected with CVS strain, but not in weanling and adult mice.

In the present issue, in an original study, M. S. Suja et al. evaluated the role of apoptosis in rabies encephalitis in humans, canines, and rodents infected with wild-type street virus and compared with a rodent model infected with laboratory-passage and -attenuated rabies virus, inoculated by different routes. They also studied the age-dependent expression of apoptosis in mice when infected with CVS

strain. Rabies viral load and encephalitic pathology were more evident in the human and canine brain in contrast to rodents infected with wild type of virus, but absence of neuronal apoptosis was common. On the contrary, as observed by other researchers, apoptosis was recorded in suckling mice infected with CVS strain, more evident by intracerebral inoculation and rarely in wild-type street virus-inoculated rodents. Interestingly the apoptotic cell signal was noted only in inflammatory cells, but was distinctly absent in neurons and glia. It is suggested that apoptotic cell loss only in inflammatory cells but not in neurons, could be a natural adoptive mechanism by the rabies virus to facilitate the survival of the virus, its propagation in stable population of neurons. It is also evident that the apoptotic cell damage is not responsible for the evolution of clinical features and terminal mortality following rabies infection. This also could account for long incubation period and long-term survival of the rabies virus in the host. It is not yet clear in which of the neuroanatomical areas in the mammalian system the virus resides dormant in latency to get activated in opportune moment. Further studies on molecular, cytokine/chemokine pathways, and neurotransmitter pathways coupled with investigating aberrant neurophysiology may offer clues to bimodal clinical manifestation and fatality from rabies virus. Among the four types of Prion disease, Creutzfeldt-Jakob disease (CJD), sporadic CJD (sCJD) is the commonest form arising from random mutation or posttranslational modification of the PrP gene. On the contrary, the new type of CJD, namely, the variant CJD (vCJD) manifests in young (mean age of 23 yrs) has longer duration of illness (12–24 months), with psychiatric presentation and absence of characteristic EEG changes. This form of CJD is causally linked to oral ingestion of meat from cattle infected with bovine spongiform encephalopathy. Neuropathologically CJD is characterized by spongiform change of cortical neuropil in cerebral cortex, cerebellar molecular layer, diencephalic nuclear areas and brainstem, neuronal loss, and reactive astrocytosis. Fairly distinct differences in the pattern of prion protein distribution recognized by immunohistochemistry are described. In sCJD, the deposition of PrPSc occurs in a synaptic pattern, distributed along the cortical ribbon and the neuropil of nuclear areas reflecting diffuse degenerative change in the presynaptic terminals and relative failure to aggregate. In the case of vCJD, the PrPSc deposits take the form of classic mature plaques of Alzheimer's disease (AD) with dense central core and less compacted halo of deposit around, and diffuse deposits (fine feathery diffuse deposits akin to immature plaques in AD) floridly.

These morphological variations appear to reflect differential distribution of the prion protein in different anatomical areas corresponding to the evolution of pathology spreading along relatively distinct axonal pathways reaching the cortical lamina and other nuclear areas. In other neurodegenerative disorders (β amyloidopathies, taupathies, and synucleinopathies) like in AD, Lewy body dementia, Pick's disease the density distribution of the pathological change in cerebral cortical ribbon varies across the different cortical lamina. This probably reflects laminar spread and distribution of the pathological changes corresponding to degeneration of specific anatomical pathways having their neurons of origin or presynaptic axonal termination in particular cortical lamina. To an extent, this laminar distribution gives insight into neuroanatomical progression of the proteinopathies in the cerebral cortex and corresponding clinical cognitive and motor abnormalities during the disease progression.

In this issue, R. A. Armstrong from Birmingham evaluated the laminar distribution of the pathological changes in sporadic and variant CJD by rigorous quantitative morphometry in well-characterized samples. The cases of sCJD were homozygous for methionine at codon 129 with Type 1 PrPSc (MIM1). All the cases of vCJD were also methionine/methionine (M/M) homozygotes at codon 129. Thus, the cases analyzed had relative genetic homogeneity to compare. The cases of sCJD revealed diffuse spongy change in the cortex affecting all the cortical lamina, more surviving neurons in upper layers of the cortex and neuronal depletion in lower layers, classical synaptic pattern of prion protein deposition, and gliosis in the lower layers. On the contrary, in cases of vCJD, the spongy change was more evident in the upper layers corresponding to subpial spongy change and presynaptic targeting axonal pathology. This was further corroborated by florid and diffuse deposits of prion protein in upper cortex. The astrocytosis in vCJD was essentially similar to sCJD, more evident in the lower layers, probably as a late event to neuronal and axonal degeneration. Similar though labour intensive, study in some of the neuroanatomical areas like cerebellum, brainstem, and diencephalic nuclei leading to neo cortex probably can offer insight into temporal evolution and neuroanatomical spread of prion pathology and contrasting features with other protein misfolding neurodegenerative disease.

In conclusion, the various articles in this issue address the pathology, pathogenesis and diagnosis of infections of CNS.

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Review Article

Intracranial *Aspergillus* Granuloma

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Intracranial fungal granulomas are rare and of the histologically verified granulomas, *Aspergillus* spp. is the commonest causative fungal pathogen. Most of the reported large series of aspergillus granulomas are from countries with temperate climate like India, Pakistan, Sudan, and Saudi Arabia. In contrast to disseminated aspergillosis that occurs in immunosuppressed individuals, most of the intracranial aspergillus granulomas are reported in immunocompetent individuals. The temperature, humidity, high spore content in the atmosphere during ploughing, and occupation as agricultural worker are implicated in the pathogenesis. The sinocranial spread is the most common route of intracranial extension. Extracerebral firm fibrotic lesions and skull base lesions are common. Extensive fibrosis and large number of multinucleated giant cells are the characteristic histological features and these pathological features have therapeutic relevance.

1. Introduction

Fungal infections of the central nervous system (CNS) are more frequently reported in the last few decades mostly due to increase in the population at risk, increased awareness, and better diagnostic modalities [1–4]. However, in the recent years there has been increase in the number of CNS fungal infections in immunocompetent individuals [2–12].

Fungi are ubiquitous in nature but have low virulence and cause disease usually when the host defenses are compromised. The fungi enter the CNS by hematogenous route from the systemic focus, or by contiguous spread from paranasal sinuses (PNS), ear or skull bone; or by direct inoculation during trauma or surgical procedure [1–5]. The pathology depends upon the route of spread, host immunity, and type of fungus, hyphae, or yeast.

The fungi may involve any part of the neuroaxis, and the pathology includes meningitis, encephalitis, abscess, granuloma, and vasculitis with associated infarction and hemorrhage and aneurysmal formation [1–4, 13]. The type of pathology, to some extent, determines the presenting clinical manifestations. This paper will discuss the experience with intracranial *Aspergillus* granuloma.

2. Epidemiology

The incidence of CNS fungal infections parallels the incidence of systemic fungal infections. The estimated annual incidences of invasive fungal infections caused by *Aspergillus* species are 12–34 [17]. The reported incidence of CNS involvement associated with invasive aspergillosis is about 4–6% [18]. Intracranial *Aspergillus* granulomas are rare space occupying intracranial lesions [2–4, 7, 9, 11, 13, 16–21] and most of the reported large series are from countries with temperate climate like India, Pakistan, Sudan, and Saudi Arabia [2–4, 6–8, 10, 12–14, 16–26].

Among the intracranial fungal granulomas, *Aspergillus* granuloma is the most commonly reported granuloma [2–4, 13, 14, 20–25] (Table 1). The prevalence of intracranial fungal mass lesions in major neurosurgical centers in India is around one to two per years [27], and *Aspergillus* spp. is the commonest causative fungal pathogen accounting for 56% to 69% of the intracranial fungal mass lesions [2, 19, 20],

Table 1: Intracranial *Aspergillus* granulomas.

	Kak et al. 1989 [14]	Camarata et al. 1992 [15]	Naim-Ur-Rahman et al. 1996 [16]	Murthy et al. 2001 [7]	Alrajhi et al. 2001 [6]	Siddiqui et al. 2004 [8]	Sundaram et al. 2006 [2]
Number	62	13	9	21	23	35	130
Age in years	12–48		26–66	19–65	9–. 61	14–74	5–75
M: F	NA	NA	1 : 08	10 : 06	14 : 09	23 : 02	1.8 : 1
Predisposing Factors	Nil	Nil	Nil	DM-2	DM-4	Nil	Nil
Route of spread	Sino-orbital 10 Hematogenous 52	Sinocranial 9 Hematogenous 4	Sinocranial 9	Sinocranial 16	Sinocranial 9	Sinonasal 35	Sinocranial 103 Hematogenous 20 Others 7
Pathology	Granulomas 28 Disseminated 34	Granulomas 12 Disseminated 01	Granulomas 9	Granulomas 16 Disseminated 5	Granulomas 23	NA	Granulomas 68
Culture	9	NA	—	*A. flavus* 4 *A. fumigatus* 2	*A. flavus* 15 *A. fumigatus* 2	15	*A. flavus* 10 *A. fumigatus* 5 *A. niger* 2 *A. terreus* 1 Sterile 3

NA: Not available, DM: Diabetes Mellitus.

Table 2: Intracranial fungal granuloma: series from temperate climate.

	Anandi et al. 1993 [25]	Santosh et al. 1996 [3]	Dubey et al. 2005 [20]	Sundaram et al. 2006 [2]
n	4/41	15/65	40	74/130
Occupation	NA	Agricultural worker	NA	Agricultural worker, Manual labourer
Organism	*Aspergillus* (4)	*Aspergillus* 10 Cryptococcus 2 Phaeohyphomycosis 3	*Aspergillus* 25 Cryptococcus 3 Phaeohyphomycosis 4 Zygomycosis 7 Candidiasis 1	*Aspergillus* 68 Cryptococcus 1 Phaeohyphomycosis 1 Zygomycosis 1 Candidiasis 2 Mixed 1

n: Number of cases, NA: Not Available.

whereas it was the causative fungus in 5% of the fungal mass lesions in the series from USA [28] (Table 2).

3. Pathogenesis

Aspergillus spp. are the most clinically significant moulds and are ubiquitous throughout the world. They are present in soil, water, decaying vegetation, and organic debris. *A. fumigatus* causes most disease followed by *A. flavus* and *A. terreus*. *A. flavus* is the commonest agent when the infection extends from PNS to CNS [1–4].

Brain is remarkably resistant to fungal infections due to the abundant blood supply and also due to the relatively impermeable blood-brain barrier. Despite the fact that the brain and subarachnoid space are protected by anatomic and functional barriers, under special conditions and immune system abnormalities, fungal pathogens breach these barriers [29]. Invasive disease is seen mostly in patients who are significantly immunocompromised: patients with prolonged neutropenia, hematological malignancies or advanced AIDS, and hematopoietic stem cell transplant and solid organ transplant [30, 31]. However, *Aspergillus* granulomas in countries with temperate climates are most commonly reported in immunocompetent individuals [2, 7, 13].

Aspergillus moulds enter the CNS by hematogenous route from the systemic focus, mostly from the lung or by contiguous spread from paranasal sinuses (PNS), ear or skull bone or by direct inoculation during trauma or surgical procedure [1–5]. The sinocranial form of CNS aspergillosis is often reported from countries with temperate climate [2, 7, 13]. In countries with temperate climate, the temperature and humidity favor the growth of the fungus. Ploughing during agriculture works or construction activities results in aerosolization of number of spores into the environment.

High spore content in the atmosphere exposes the agriculture workers and workers in the construction activity to inhale the fungal spores.

The spores are colonized in the lungs, nose, PNS, mastoid air cells, and ear canal. Closed cavity and anaerobic atmosphere promote growth of the fungus. There may be local

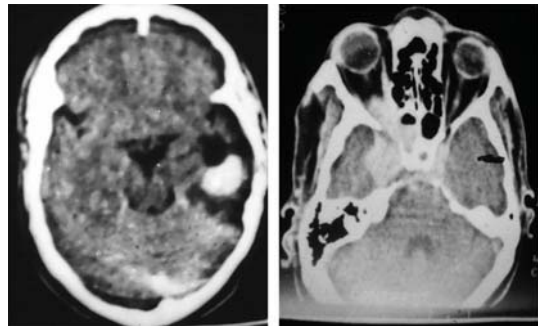
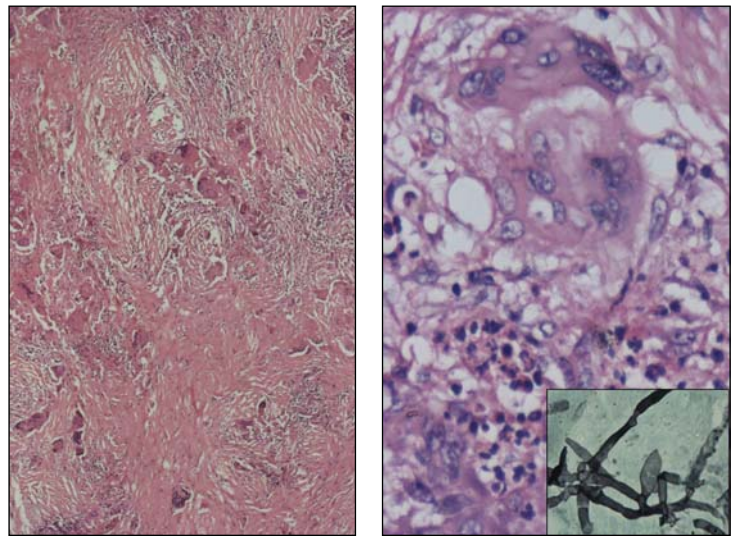


Figure 1: Contrast CT scans showing densely enhancing left temporal intraparenchymal *Aspergillus* granuloma (a) and enhancing mass lesion in the ethmoid sinuses, right orbital apex, right extraparenchymal temporall fossa and left cavernous sinus skull base *Aspergillus* granuloma (b).



(a) (b)

Figure 2: Histological sections of *Aspergillus* granuloma. (a) Giant cell rich granulomas with dense fibrosis (H&E; X40). (b) Giant cell with intracytoplasmic negative staining hyphae (H&E; X200). Inset: Gomori's silver methenamine stain highlighting slender septate hyphae of *Aspergillus* spp. (GMS; X400).

altered immunity which promotes the mucosal invasion of the fungus [1–4]. The immunopathogenesis of CNS fungal infections remains incompletely studied, with most of the knowledge coming from studies on experimentally infected animals. The activation of brain resident cells such as microglia, astrocytes, and endothelial cells combined with relative expression of immune-enhancing and immune-suppressing cytokines and chemokines may play a determinant role in immunopathogenesis [29].

4. Pathology

Mostly these lesions, because of the sinocranial spread of the infection, are extraparenchymal and skull base in location. Skull base location includes anterior and middle cranial fossae, orbit, orbital apex, cavernous sinus, and rarely posterior fossa (Figure 1). Rarely these lesions can be primarily intraparenchymal, involving frontal and temporal lobes [19, 21]. The location of the lesions probably explains the clinical syndromes. Histologically the granulomas show dense fibrosis and an infiltrate of lymphocytes, plasma cells, and mononuclear cells. The multinucleate giant cells are foreign body type and contain slender septate, acute angle branching hyphae of *Aspergillus* spp. The extracerebral granulomas differ from intraparenchymal granulomas in having extensive fibrosis [19–21]. *Aspergillus* granuloma on haematoxylin and eosin staining sometimes may be mistaken for tuberculous granuloma. However, the prominence of multinucleated giant cells with admixture of neutrophils, plasma cells and eosinophils and relatively less number of epitheloid cells differentiates tuberculous granuloma from *Aspergillus* granuloma. Good scanning of the biopsy may reveal fungal hyphae within giant cells.

Gomori's methenamine silver (GMS) and Periodic Acid Schiff (PAS) stains demonstrate the slender septate hyphae with acute angle branching of the *Aspergillus* spp. in

Aspergillus granuloma [19–21] (Figure 2). The extensive fibrosis observed in the extraparenchymal, sinocranial *Aspergillus* granulomas has therapeutic relevance. Extensive fibrosis does not allow effective penetration of systemically administered antifungal agents. Thus these lesions need extensive radical excision to achieve cure [16, 19]. The other approach to achieve effective therapeutic concentration of the antifungal agents will be intralesional administration of the antifungal agents by Ommaya reservoir [16, 19]. The pathology of haematogenous dissemination to CNS, because of angioinvasive character of *Aspergillus*, includes ischemic infarction and haemorrhage, and the pathology in the sinocranial aspergillosis is characterized by well-formed granuloma [2].

5. Sinocranial *Aspergillus* Granuloma—Pathological Features—Therapeutic Relevance

A. fumigatus elaborates a substance called fumagillin which is responsible for fibrosis [13]. Many allergens present in *A. fumigatus* are present at high levels of homology in *A. flavus*. *A. flavus* causes majority of the sinocranial infections. *A. flavus* produces many more allergic proteins than the two currently known proteins (Asp fl 13 and Asp fl 18) and may possess an allergen component similar to that of *A. fumigatus* [35]. *A. flavus* seems to be more virulent and more resistant to antifungal drugs than most of the other *Aspergillus* species.

The extensive fibrosis observed in the extraparenchymal, sinocranial *Aspergillus* granulomas has therapeutic relevance. Extensive fibrosis does not allow effective penetration of systemically administered antifungal agents. Thus these lesions need extensive radical excision to achieve cure [8, 16, 19, 30, 32]. The other approach to achieve effective therapeutic concentration of the antifungal agents will be intralesional administration of the antifungal agents by Ommaya reservoir [7, 15, 33].

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Research Article

Microbiological Spectrum of Brain Abscess at a Tertiary Care Hospital in South India: 24-Year Data and Review

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Intracranial abscesses are life-threatening infections that pose a diagnostic challenge not only to the neurosurgeon but also to the microbiologists. Detailed studies documenting the spectrum of infecting agents involved in brain abscesses are limited from India. *Materials and Methods*. This is a retrospective analysis of 352 samples from 1987 to 2010 analyzed at a tertiary care hospital in South India from 1987 to 2010, to document the changing trends with time. *Results*. The age of the patients ranged from 2 to 80 years, a larger number of males being affected. Otogenic infections were the most common cause while cryptogenic abscesses were 20%. Gram stain and culture positivity were 78% each. Gram-positive and negative facultative aerobes and obligate anaerobes were also on the rise. Unusual organisms, like *Burkholderia pseudomallei, Salmonella typhi, Nocardia species, Cladosporium bantiana, Fonsecaea pedrosoi, Entamoeba histolytica, and Acanthamoeba* were also isolated and/or detected from the brain abscesses aspirate or resected tissue. *Summary*. New and emerging pathogens associated with brain abscess, especially in immunosuppressed individuals, have renewed the necessity of an early detection, and it will be of great value in appropriate management of patients with brain abscess.

1. Introduction

Intracranial abscesses (usually referred to as brain abscess), though uncommon in developed countries, are serious, life-threatening infections [1–3]. Major advances, such as stereotactic neurosurgical procedures, discovery of newer antibiotics, especially metronidazole against anaerobes and ceftriaxone which effectively crosses the blood brain barrier, and newer imaging techniques for early detection of brain abscesses [3], have lead to a substantial reduction in the mortality [4, 5]. Despite these advances, brain abscess remains a potentially fatal central nervous system (CNS) disease, especially in developing countries [3–9].

Difficulties in the diagnosis of intracranial abscess are mainly due to protean clinical manifestations and similarities in the imaging and morphologic appearance of some intracranial mass lesions, like cystic gliomas and metastases. The frequent delay in making the diagnosis renders this condition a significant challenge for the neurosurgeon [8–10] in the management of the case.

New and emerging pathogens, especially in immunosuppressed individuals, have renewed concern about the diagnosis and treatment of brain abscess and equally pose a challenge to the clinical microbiologist [1, 3, 7, 11]. Meticulous microbiological investigations, including critical microscopic examination for all possible infectious agents and employing detailed microbial investigative armamentarium for the isolation of the organism(s) from the abscess material and from the probable primary site of origin of the infection elsewhere in the body, will definitely aid in the identification of the etiological agent(s) [2, 9–11]. These findings will enable the neurosurgeon and the infectious disease (ID) specialist to treat the brain abscess more rationally and appropriately [5, 7, 8, 10].

It is well worth stating that a brain abscess is not only a neurosurgical emergency but also a microbiological emergency and a diagnostic challenge to both the disciplines [1, 10, 12].

The objectives of this retrospective study were to analyze the microbiological findings in the purulent aspirates and/or tissue obtained from the brain abscesses and discuss the changing and evolving spectrum of infectious agents observed over the past 24 years with a brief review of other studies.

2. Materials and Methods

This is a hospital-based retrospective microbiological analysis of 352 brain abscess materials (purulent aspirates and/or tissue) that were received between 1987 and 2010, by the Microbiology Laboratory, at the Nizam's Institute of Medical Sciences, a tertiary care and a teaching hospital, in South India. The data was analyzed in two groups—group I, between 1987 and 1993 (published data) [12] and group II between 1994 and 2010, to document the changing trends in the microbial flora and treatment strategies.

(1) The demographic and clinical information of the patients was retrieved from the medical records section. Relevant data recorded included the age and sex of the patients, intracranial location of the abscess(es), the probable primary source of the infectious agents leading to the formation of the abscess(es).

(2) The microbiology data was retrieved from the microbiology records maintained as a database in the Microbiology Department.

(3) Wherever the resected brain abscess tissue was received, the histopathological features were corroborated to complement the culture results.

3. Microbiological Investigations

Specimen collected from a brain abscess (either through a burr hole or craniotomy) during any time of the day and submitted for microbiological investigations was considered as an emergency specimen and was processed immediately in the microbiology laboratory, on priority.

Specimen received between the years 1987 and 1993 (study group I) [12] was processed only for bacteria (aerobic and anaerobic), by routine microbiological procedures [13].

(i) Gram's stain was carried out on all specimens for bacteria, while Zeihl Neelsen's (ZN) stain for acid fast bacilli (AFB) was done in only one case of tuberculous brain abscess.

(ii) Aerobic cultures were performed on 7% sheep blood agar and McConkey agar and incubated at 37°C for 48 hours, before being declared as sterile. All positive cultures were further processed for identification and antibiotic susceptibility patterns [13].

(iii) Anaerobic culture was performed on 7% sheep blood agar plates and incubated in the Dynamicro Gaspak system. Metronidazole disc (5 μg) was placed to observe for antibiotic susceptibility of anaerobes. Isolates susceptible to metronidazole were considered to be anaerobes. Gram's stain of such isolates was carried out to confirm the morphology and the genus of the isolate. Aerotolerance test was performed to demonstrate that these isolates were obligate anaerobes.

(iv) The specimen from the tuberculous abscess showed AFB on ZN stain. The material was inoculated on Lowenstein Jensen's (LJ) medium, and the isolate was identified as *Mycobacterium tuberculosis (M.tb)* based on its rate of growth and susceptibility to para nitro benzoic acid [12].

The rest of the 302 brain abscess specimens (study group II) were processed for detection of bacteria (aerobes and anaerobes), *mycobacteria*, fungal pathogens including *Nocardia* and *Actinomycetes,* and parasites, by standard microbiological procedures [13] and by semiautomated identification systems (Mini API and the Vitek 2, bioMérieux, USA). Various detection procedures used were as per standard guidelines [13]. The anaerobic isolates were identified up to the genus level only.

4. Results

The average number of brain abscess specimens received for microbiological analysis was 21 per year.

Brain abscesses were diagnosed in all decades of life, with the ages ranging from 2 years to 80 years (mean of 28.5 ± 17.6) and a male preponderance (male to female ratio was 2.7 : 1). The youngest patient (2 years) developed a frontal lobe abscess with an underlying septicemia and was referred to our institute for further management. The oldest patient was an 80-year-old lady, with chronic suppurative otitis media (CSOM) and a parietal lobe abscess.

Table 1(a) shows a comparison between the two study groups in terms of the location and source of infection of the brain abscess. Tables 1(b) and 1(c) highlight the topographic distribution of the abscesses and the probable source of infection documented in study group II.

There were 310 (88.1%) nontraumatic and 42 (11.9%) posttraumatic brain abscesses in our study. Though solitary abscesses (313/352; 88.9%) were more common, multiple abscesses (39/302; 12.9%), predominantly otogenic, were noted only in the study group II. There were 6 cases of subdural empyema and 3 cases of extradural abscesses. The rest of the brain abscesses were intracranial, involving the brain parenchyma. The majority of these intracerebral abscesses were located in the parietal region 102/352 (28.9%), followed by the frontal and the temporal lobes (21%).

5. Microbial Spectrum

Staining and microscopy of the brain abscess material (pus and/or tissue) revealed the pathogens in 41/50 (82%) [12] and 221/302 (73.1%) of the cases in the 2 groups, respectively. In the rest of the cases, only polymorphonuclear cells

Table 1: (a) Neuroanatomical location and source of infection of brain abscess comparison between the two study groups. (b) Neuroanatomical location and source of infection of solitary brain abscess in study group II ($n = 263$). (c) Location and source of infection of multiple brain abscesses in study group II ($n = 39$).

(a)

	Primary source of infection											
Location	CSOM		CHD		Pulm		Trauma		Cryptogenic		Total	
Group	I	II	I	II	I	II	I	II	I	II	I	II
Parietal	8	4	4	11	4	1	1	23	5	23	22	62
Frontal	4	20	2	3	—	1	—	6	2	33	8	63
Temporal	6	46	1	3	—	—	—	3	2	11	9	63
Cerebellar	4	15	—	—	—	1	—	—	—	2	4	18
Occipital	2	5	1	1	—	—	—	—	1	2	4	8
Subdural	—	1	1	—	2	—	—	—	—	—	3	1
Total	24	91	9	18	6	3	1	32	10	71	50	215

CSOM: chronic suppurative otitis media, CHD: congenital heart disease, and Pulm: pulmonary.
Group I: 1987–1993 and Group II: 1994–2010.

(b)

	Primary source of infection											
Location	ASOM	CSOM	PNS	CHD	Sepsis	Odont	Pulm	Trauma	Op trauma	Misc	Crypto	Total
Parietal	—	4	—	11	17	1	1	9	14	1	23	80
Frontal	—	20	6	3	4	—	1	2	4	—	33	73
Temporal	—	46	2	3	3	1	—	1	2	3	11	72
Cerebellar	1	15	—	—	1	—	1	—	—	2	2	22
Occipital	—	5	—	1	—	—	—	—	—	—	2	8
Subdural	—	1	—	—	2	—	—	—	—	—	—	3
Epidural	—	—	—	—	3	—	—	—	—	—	—	3
Thalamus	—	—	—	—	—	—	—	1	—	—	—	1
Medullary	—	—	—	—	—	—	—	—	—	—	1	1
Total	1	91	8	18	30	2	3	13	20	6	71	263

ASOM: acute suppurative otitis media, CSOM: chronic suppurative otitis media, PNS: paranasal sinusitis, CHD: congenital heart disease, Odont: odontogenic, Pulm: pulmonary, Op trauma: operative trauma, Misc: miscellaneous, and Crypto: cryptogenic.
E. hystolytica and Acanthamoeba cases not included in analysis.

(c)

Location	ASOM	CSOM	PNS	CHD	Sepsis	Odont	Pulm	Trauma	Op trauma	Misc	Crypto	Total
FP	—	5	—	1	1	—	—	3	—	—	—	10
FT	—	2	—	1	—	—	—	1	—	—	—	4
FO + SD	—	1	—	—	—	—	—	—	—	—	—	1
F + SD	—	—	—	—	1	—	—	—	—	—	—	1
PO	—	5	—	3	—	—	—	1	—	3	—	12
TP	—	5	—	—	1	—	—	3	—	—	1	10
T + SD	—	1	—	—	—	—	—	—	—	—	—	1
TOTAL	—	19	—	5	3	—	—	8	—	3	1	39

CSOM: chronic suppurative otitis media, PNS: paranasal sinusitis, CHD: congenital heart disease, Odont: odontogenic, Pulm: pulmonary, Op trauma: operative trauma, Misc: miscellaneous, Crypto: cryptogenic, FP: frontoparietal, FT: frontotemporal, FO: fronto-occipital, SD: subdural, F: frontal, PO: parieto-occipital, TP: temperoparietal, and T: temporal.

were seen, indicating an inflammatory process. Microscopy was positive on all the fungal, *mycobacterial*, *Nocardial,* and parasitic abscesses. However, the microscopy was negative in 12 bacterial culture-positive cases in group II.

Microbiological cultures for bacteria, *mycobacteria,* and fungi (included in the study group II only) revealed the etiological agent(s) in 44/50 (88%) [12] and 203/302 (67.2%) cases in the two groups. The spectrum of organisms isolated from the brain abscesses in both of the groups is compared in Tables 2(a), 2(b), and 2(c).

Gram-positive facultative aerobes were isolated more frequently than the gram-negative aerobes. Among the isolates, 35/44 (79.5%) and 158/203 (77.83%) were facultative aerobes, 7/44 (15.9%) and 32/203 (15.8%) were obligate

Table 2: (a) Single and polymicrobial aerobic bacterial isolates from brain abscess. (b) Single and polymicrobial anaerobic isolates from brain abscess. (c) Miscellaneous bacterial and fungal isolates from brain abscess.

(a)

Type of organism	No. of isolates	
	Group I ($n = 35$)	Group II ($n = 158$)
Gram-positive facultative aerobes		
Staphylococcus aureus	**11**	**51**
Enterococci species (avium, faecium, and faecalis)	—	**32**
Beta haemolytic Streptococci	**5**	**17**
Alpha haemolytic Streptococci	**3**	**5**
Streptococcus pneumoniae	**1**	**3**
Diphtheroids	—	**1**
Gram-negative facultative aerobes		
Escherichia coli	**1**	**9**
Klebsiella pneumoniae	**2**	**10**
Enterobacter species	**2**	**6**
Proteus species	**5**	**1**
Morganella morganii	—	**1**
Citrobacter freundii	—	**2**
Pseudomonas aeruginosa	—	**5**
Acinetobacter species	—	**4**
Salmonella typhi	—	**1**
Burkholderia pseudomallei	—	**1**
Neisseria meningitides	—	**1**
Total	**30**	**150**
Facultative aerobes-polymicrobial		
Klebsiella + Proteus	**2**	—
BH Streptococci + Proteus	**1**	—
S. aureus + Proteus	**2**	—
Pseudomonas + Klebsiella	—	**1**
Klebsiella + E. coli	—	**1**
Klebsiella + S. aureus	—	**1**
Pseudomonas + S. aureus	—	**1**
Klebsiella + Acinetobacter + Enterococci	—	**1**
S. aureus + Acinetobacter	—	**1**
Enterococci + Strep. pneumonia	—	**1**
S. aureus + E. coli	—	**1**
Total	**5**	**8**

(b)

Type of organism	No. of isolates	
	1987–1993 ($n = 7$)	1994–2010 ($n = 32$)
Gram-positive obligate anaerobes		
Peptococcus species	—	**3**
Peptostreptococcus species	**3**	**11**
Gram-negative obligate anaerobes		
Bacteroides fragilis	**1**	**3**
Veillonella	—	1
Total	**4**	**18**

(b) Continued.

Type of organism	No. of isolates	
	1987–1993 ($n = 7$)	1994–2010 ($n = 32$)
Mixed anaerobic isolates		
Peptostreptococcus + B. fragilis	—	2
Prevotella melaninogenicus + Peptostreptococcus	—	1
Total	—	**3**
Mixed aerobic and anaerobic isolates		
Pseudomonas + B. fragilis + Peptostreptococcus	—	2
Strep. pneumonia + Proteus + B. fragilis	1	—
Pseudomonas +B. fragilis	—	2
S. aureus + Peptostreptococcus	—	1
Proteus + Peptostreptococcus	—	**2**
S. aureus + B. fragilis	1	1
BH Streptococci + B. fragilis	**1**	**1**
Pseudomonas +Veillonella	—	**1**
Veillonella+ Providencia stuartii	—	**1**
Total	**3**	**11**

(c)

Type of organism	No. of isolates	
	1987–1993 ($n = 2$)	1994–2010 ($n = 13$)
Mycobacterial and *Nocardial* isolates from brain abscess		
Mycobacterium tuberculosis	1	6
Mycobacterium fortuitum	1	—
Nocardia brasiliensis	—	1
Nocardia asteroides	—	1
Fungal isolates from brain abscess		
Nocardia asteroides + Aspergillus terreus	—	1
Aspergillus flavus	—	1
Aspergillus flavus + BHS	—	1
Cladosporium bantiana	—	1
M. tb + Fonsecaea pedrosoi	—	1
Total	**2**	**13**

E. histolytica and Acanthamoeba cases not included in analysis.

anaerobes, and 2/44 (4.5%) and 7/203 (3.4%) were *mycobacteria*. Nocardia species 3/203 (1.5%) and fungal isolates 5/203 (2.5%) were seen only in group II, probably due to the extensive methods employed. The obligate anaerobes were isolated mainly from the brain abscesses originating from the ear, paranasal, and oral infections. Mixed aerobic and anaerobic abscesses were also frequently encountered. There were 30 microscopy-positive abscesses with very small Gram-positive cocci in pairs and or chains, on Gram's stain, resembling anaerobic cocci. However, no organisms could be recovered by any of the culture methods used from these specimens.

5.1. Bacterial Isolates (Tables 2(a) and 2(b)).

5.1.1. Facultative Aerobes (180 Single and 13 Mixed Aerobes, Total 193) (Table 2(a)). Staphylococcus aureus (S. aureus) (68/192, 35.2%) was the most common isolate, often as a single isolate. Compared to the group I, the incidence of *S. aureus* infections was about 4 times more (Table 2(a)). These isolates were usually from brain abscesses associated with CSOM and trauma cases, in the study group II. There were 6/55 methicillin-resistant *S. aureus* (MRSA) isolates, from brain abscesses following a road traffic accident (RTA), and they were sensitive to vancomycin. The rest of the isolates were methicillin-sensitive *S. aureus* (MSSA) and sensitive to a broad range of antibiotics.

Enterococcus species (34/193, 17.6%) were isolated frequently from the cases with CSOM. Compared to the study group I, *Enterococcus* species appeared to be significant emerging pathogens of brain abscess at our centre. The species isolated were *E. avium*, *E. faecalis*, and *E. faecium*. All the isolates demonstrated a high susceptibility to beta-lactams and vancomycin. The high rates of isolation could

be due to the use of chromogenic media for primary culture of the pus and automated identification systems like the Vitek 2.

Beta haemolytic Streptococci (22), *alpha haemolytic Streptococci* (8), *and S. pneumoniae* (5) were mostly otogenic and were highly susceptible to penicillin and other betalactams. The isolation of these important pathogens was more in the study group II, often as single isolates.

Aerobic Gram-negative bacilli (62/193, 32.1%) though mainly otogenic, these organisms were also frequently isolated from the postcraniotomy and hematogenic abscesses. The enteric bacilli, such as *Escherichia coli (E. coli), Klebsiella pneumoniae (K. pneumoniae), Enterobacter* species, *Proteus* species, *Morganella morganii,* and *Providencia stuartii* were the frequent isolates. Like the Gram-positive pathogens, the incidence of the Gram-negative bacilli, especially of multidrug-resistant and nosocomial pathogens, was seen to increase. *E. coli and K. pneumoniae* were highly resistant organisms producing extended-spectrum β-lactamase (ESBL). Nosocomial pathogens such as *Pseudomonas aeruginosa (P. aeruginosa)* and *Acinetobacter baumannii (A. baumannii)* were also isolated.

5.1.2. Obligate Anaerobes (22 Single and 17 Mixed, Total 39). An increasing incidence of polymicrobial anaerobic abscesses was documented. The spectrum of anaerobes in this study is shown in Table 2(b).

5.1.3. Uncommon Gram-Negative Bacilli

(i) *B. pseudomallei* (2 cases): One patient with melioidosis had presented with fever and abdominal pain and developed calvarial osteomyelitis and multiple parietal and sagittal sinus abscesses [14]. Another patient with a splenic abscess due to *B. pseudomallei* developed a frontal lobe brain abscess. However, the organism could not be isolated on culture from the brain abscess pus.

(ii) *S. typhi* was isolated from the brain abscess pus in a case of septicemia.

5.1.4. Mycobacterial Species (8/352, 2.2%). All the 8 TB abscesses revealed AFB on microscopy, and *M. tb* was isolated by culture on LJ Medium (study group I) and BACTEC 460 TB system (Becton Dickinson, USA). In our study group I, two cases of *mycobacterial* abscesses, (*M. tb* (1) and *M. fortuitum* (1) [12] were documented, while there were 6 cases of tuberculous brain abscesses and one was a mixed fungal and TB abscess [15], in the study group II.

5.1.5. Nocardial Species (3/302, 1%). There were 2 parietal lobe abscesses caused by *N. asteroides* in our group (one in a postrenal transplant patient and the other in a case of atopic dermatitis on steroids). We also reported a case of a young female with mycetoma on the back, who presented with an epidural abscess. *N. brasiliensis* was isolated from the sinuses on the skin and from the epidural abscess pus [16].

5.2. Fungal Agents. There were 5 (1.7%) cases of fungal brain abscess in our series, that occurred in various clinical settings. The cases with *Aspergillus* abscesses had a fatal outcome.

We reported a case of a 23-year-old immunocompetent male who sustained a head injury and developed a capsuloganglionic region abscess due to the neurotropic fungus, *Cladophialophora bantiana* [17]. Diagnosis was made based on the microscopy, mycology, and histopathology findings of the aspirate obtained from the abscess. The patient responded clinically to amphotericin B.

5.3. Protozoa

(a) *E. histolytica* abscess: the trophozoites of *E. histolytica* were demonstrated on microscopy in a patient with cerebral amoebiasis. There was no evidence of diseases elsewhere. Patient had a complete recovery following a total excision of the abscess and Metronidazole therapy [18].

(b) A fatal case of an acute frontal lobe abscess due to *Acanthamoeba,* in an immunocompetent patient with meningoencephalitis, was documented. The cerebrospinal fluid (CSF) from the patient showed the *Acanthamoeba* (polygonal) cysts on Gram stain and calcofluor white stain. However, a coculture with *E. coli* was unsuccessful in isolating the organism [19].

6. Discussion

Brain abscesses have been well known and reported from the beginning of the Hippocratic era [10, 11]. Essentially, a brain abscess is a focal intraparenchymal collection of pus and is classified based on the anatomical location or the etiologic agent causing it. It begins as a localized area of cerebritis and evolves into a collection of pus surrounded by a vascularized capsule [5, 20].

Our data shows that the incidence of brain abscess continues to be significant in the neurosurgical clinical setting. The average incidence of brain abscess among all the space-occupying lesions in our institute is about 25%. Recent studies from India have reported an average of 9 to 15 cases per year [1, 7, 8], while studies from developed countries recorded a lower number, 5–12 cases per year [10, 21, 22]. As per our data and other studies, brain abscesses occur in all decades of life, with a male preponderance [1, 6, 21, 22]. However, the reasons for the male preponderance, are not clear [1].

The spectrum of organisms seen in brain abscess usually depends on the primary source of infection. Gram-positive cocci, especially the *Enterococci*, and Gram-negative bacilli, especially *E. coli* and *K. pneumoniae,* were more frequently isolated in group II than in the earlier group I. This is probably due to the use of the Bact/alert system and direct inoculation of the purulent aspirates into the bottles. A larger number of antibiotic-resistant Gram-negative bacilli were isolated from cases in group II, probably due to the increased number of trauma cases in this group.

6.1. Otogenic Abscesses. CSOM continues to be the most frequent predisposing condition in all age groups (24/50 (48%) and 118/302 (39%) in both the groups), respectively, time groups. Other studies from India also documented CSOM as a major source of brain abscess, 49% [1], 31.4% [7], and 40% [8]. Though middle ear suppurative disease was seen to extend to temporal lobe or cerebellum [1, 23, 24], multiple otogenic abscesses, often involving the frontal and parietal lobes, were seen in the study group II, probably due to arterial dissemination of infective emboli. Direct extension may also occur through osteomyelitis in the posterior wall of the frontal sinus, sphenoid, and ethmoid sinuses. This direct route of intracranial extension is more commonly associated with subacute and chronic otitic infection and mastoiditis than with sinusitis [4]. Frontal or ethmoid sinus infections generally spread to the frontal lobes. Odontogenic infections can spread to the intracranial space via direct extension or a route, generally to the frontal lobe [4].

All the otogenic abscesses, in our study, were of bacterial etiology (aerobic and/or anaerobic), with culture positivity of 85%. Gram-positive facultative aerobes were the most frequent isolates from these abscesses**,** as also recorded in other studies [1, 6, 7, 23, 24]. An unusual case of concomitant TB *(M. tb)* and fungal *(Fonsecaea pedrosoi)* infections involving the middle ear cleft extending and destroying the craniovertebral junction at the skull base was successfully managed and treated at our centre [15]. Our study and others from India clearly suggest that middle ear infections need to be treated aggressively to reduce the incidence of brain abscesses [1, 24].

6.2. Odontogenic Abscesses. The oral and dental florae, mainly from the subgingival sites, are documented to be frequent sources of brain abscesses [25]. These sites are usually comprised of *Streptococcus milleri* and the *Gram negative anaerobic bacilli.* Two cases of odontogenic abscesses were noted in the study group II caused by *S. aureus* and *Prevotella melaninogenica.*

6.3. Cardiogenic Abscesses. Cyanotic congenital heart diseases such as tetralogy of Fallot (10/23, 43.5%), transposition of the great vessels (3/23, 13%), and dextrocardia (2/23, 0.08%) are documented as risk factors for brain abscess [1, 8, 26, 27] and congenital pulmonary arteriovenous malformations [28]. We documented cardiogenic abscesses in 18% (9/50) in the study group I [12] and 7.6% (23/302) in the study group II. The age of the patients with cardiogenic abscess was less than 20 years though there was one 45-year-old patient with an *S. aureus* abscess.

Patients with cyanotic heart disease have a right to left shunt of venous blood in the heart, bypassing the pulmonary circulation, thus leading to bacteremia, septicemia, and infective thromboembolism, usually in the brain. These patients also have low-perfusion areas in the brain due to chronic hypoxemia and secondary polycythemia. The abscesses, often multiple, occur at the junction of the gray and white matter. The parietal lobes are most commonly affected due to the large caliber and direct continuation of the middle cerebral artery [9]. The congenital arteriovenous anomalies become a nidus for the organisms, especially Gram-positive ones, spread to the brain leading to the development of brain abscess [1, 9]. Nineteen of the 23 (82.6%) cardiogenic abscesses in our series were positive for bacteria by microscopy and Gram-positive cocci, including facultative aerobes, and obligate anaerobes were isolated.

6.4. Traumatic Abscesses. Trauma to the skull, either following road traffic accident (21/302; 7.2%) or a craniotomy (20/302; 6.6%), is an important risk factor in the study group II, which was not encountered in the study group I, probably related to increase in speeding vehicles and availability of neurosurgical facilities. Thirty six of the forty one abscesses were solitary, and the parietal lobe was the most commonly affected site.

Microscopy revealed organisms in 92.7% abscesses, while 80.5 of them were culture positive. *S. aureus* infection was common in the RTAs, probably due to the direct implantation of the organism derived from the normal flora of the calvarial skin. On the other hand, the postcraniotomy abscesses had a spectrum that is predominantly of gram-negative facultative aerobes and was often a nosocomial infection. These isolates, most commonly *E. coli* and *K. pneumoniae,* had a high level of resistance to antibiotics. Strict aseptic measures during the postoperative management of the surgical wound and care of the intravascular catheters in these patients is very important and cannot be underscored [6, 11].

Postcraniotomy brain abscesses due to *C. bantiana* were reported by other centers [29–31]. The portal of entry of the fungus is either due to direct inoculation following trauma to the skull, either following RTA or craniotomy, inhalation of the spores, or a haematogenous spread to the brain [31].

6.5. Hematogenic Abscesses. Septicemia and/or sepsis elsewhere in the body is a probable cause for the spread of the organism from the primarily involved organ to the brain. We documented 10.9% hematogenic abscesses in the study group II. Twelve of the abscesses (36.4%) had no detectable organisms on microscopy and were sterile on culture. Menon et al. recorded 40% sterile abscesses in patients on prior antibiotics [1].

Also calling metastatic abscesses from a remote site, the hematogenic abscesses are the commonest type in developed countries [3]. They are often multiple and typically occur at the junction of the white and gray matter, where the capillary blood flow is the slow [9]. They are more commonly seen along the distribution of the middle cerebral arteries and the parietal lobes, where the regional blood flow is the highest. The common systemic sources of infection are chronic pulmonary infections, skin pustules, bacterial endocarditis, and osteomyelitis. Those with a right to left vascular shunt as a result of congenital heart disease or pulmonary arteriovenous malformations are particularly susceptible. Being hematogenous, any lobe of the brain can be affected, and some of them could be multiple [9, 28]. Though these abscesses are reported to contain a mixed flora [32],

in the present series, the infected hematogenic brain abscesses were monomicrobial, since the septicemia is usually monomicrobial. Gram-negative pathogens were more frequently isolated from these abscesses, though unusual Gram-negative organisms such as *S. typhi and B. pseudomallei* were isolated from the hematogenic brain abscess pus.

6.6. Preexisting Pulmonary Lesions. Primary infections or secondarily infected pulmonary cavities can be a predisposing cause of brain abscess, especially in the immunocompromised patients [4]. Six cases of brain abscesses of a probable pulmonary origin were recorded in group I which include 1 *M.tb,* 1 *M. fortuitum,* and 4 bacterial abscesses [12]. In the study group II, there were 3 abscesses of proven pulmonary origin (organisms were isolated from the respiratory tract), which grew *N. asteroides* (a case of postrenal transplant) and *M.tb* (2 cases), respectively.

6.7. Urosepsis. The urinary tract infections are an important primary source of brain abscess. We documented an abscess in lower midbrain in a patient with urosepsis. The abscess was sterile, probably due to prior antibiotic therapy. A similar report of brain abscess due to urosepsis was reported in literature [33].

6.8. Intracranial and Meningeal Lesions. Brain abscess has been documented to occur following a contiguous spread from infected foci within the brain or meninges or a secondary infection of preexisting intracranial lesions [4, 33]. Two brain abscesses from a primary infective focus within the brain or the meninges were documented in our study. One was a case of neurocysticercosis developing a temporal abscess due to *Neisseria meningitides,* probably a secondary infection of the cystic lesion. The other case was a case of pyogenic meningitis developing a frontoparietal abscess due to *Peptostreptococcus* species.

6.9. Osteomyelitis of the Calvarium. Osteomyelitis of the skull bones can be a primary focus with a contiguous spread to the underlying parenchyma [4] as noted in our study [14]. We documented an unusual case of *B. pseudomallei* calvarial osteomyelitis that had spread to both the parietal lobes and medially extended up to the sagittal sinus [14]. There were two other cases of calvarial osteomyelitis with sterile abscesses in the study group II.

6.10. Immunosuppression. Immunosuppression can predispose patients to the development of brain abscesses. With the increasing number of transplant surgeries, especially renal, in the recent times, it is a significant emerging problem [9]. Immunosuppression induced by steroids also may predispose the patients in developing brain abscess.

The spectrum of organisms noted in the immunosuppressed patients may not be found in immunocompetent individuals, and because of this, empirical therapy in these patients should be avoided [9]. Since, the imaging features of the abscess on computerized tomography or magnetic resonance imaging (MRI) also do not help in the diagnosis, attention should be directed to obtaining a microbiological diagnosis, whenever possible, so that appropriate antimicrobial therapy can be initiated without delay. The pus obtained from the abscess should be subjected to microbiological examination for fungal elements, AFB including *Nocardia* and parasites besides the routine aerobic and anaerobic cultures [1, 7, 9]. These compromised hosts with impaired T-lymphocyte or macrophage function are prone to develop infections with intracellular pathogens such as fungi (particularly *Aspergillus* species) and bacteria like *Nocardia* species [6, 11, 34], especially emerging *Nocardial* species (*N. cyriacigeorgica*) [35].

Brain abscess was documented in 3 cases of renal transplantation. The aspirated materials on microscopy, and culture revealed growth of *A. flavus, N. asteroids,* and *S. pneumoniae* along with *S. aureus* in the 3 cases, respectively (Table 2(c)). Another fatal case of mycotic brain abscess in a case of atopic dermatitis on immunosuppressive therapy with steroids in a 5-year-old female child and multiple parietal abscesses secondary to *A. terreus* and *N. asteroides,* probably originating from the ears or the lungs, were noted. Dias et al. documented a case of *Nocardial* brain abscess in an elderly male with undetected diabetes mellitus [34, 36].

6.11. Cryptogenic Brain Abscesses. This group comprises of those abscesses where there would be no obviously demonstrable primary focus of infection elsewhere in the body nor any underlying predisposing condition leading to infection. The reported incidence of such cryptogenic abscesses, as per several studies ranges between 15 and 22% [1, 10, 34]. The organisms in the cryptogenic abscesses have been shown to be derived from the upper respiratory tract and oral flora, comprising mainly of *Streptococcal* species and anaerobic *cocci* [1, 10]. In one of the studies, a patent foramen ovale was identified by echo cardiogram and was proposed as a possible way of migration and seeding of the oral flora to the brain [37].

We documented 23.3% brain abscesses as cryptogenic. Though the majority were solitary and sterile abscesses (53/82, 64.6%), the culture-positive cryptogenic abscesses (19/82, 26.4%) were caused mainly by a single Gram-positive facultative aerobe (12/19), among which *S. aureus* (MSSA) was the predominant isolate. The remaining cases were polymicrobial.

7. Microscopy versus Culture

The sensitivity of microscopy depends on the number of organisms in the specimen (10^3 CFU/mL). In our study, the Gram's stain was positive in 30 culture-negative cases, while it was negative in 12 culture-positive cases. There is a need to adopt methods to improve the detection rates especially by microscopy, which includes fluorescent staining using acridine orange (sensitivity (10^2 CFU/mL)) [13], calcoflour white for fungal filaments, and by auramine rhodamine fluorescent stain for *mycobacteria*.

8. Culture for Various Organisms

All the culture methods used in our study were to optimize the isolation of the various possible and cultivable etiologic agents. It has been shown that a direct inoculation and use of automated methods of culturing the pus, immediately after its aspiration, enhances the yield of organisms [7, 38]. Direct inoculation of the pus specimen into a standard anaerobic BacT/Alert bottle (bioMérieux, USA) facilitated early and better yield of the organisms. The unvented, bottled anaerobic medium facilitates the growth of both facultative aerobes and the obligate anaerobes, and the yield is further enhanced by the shaking incubator, inbuilt in the bacT/alert system [38].

Despite the meticulous conventional microbiological procedures, 6/50 (12%) and 99/302 (32.8%) abscesses were sterile, in our group, respectively. The reported incidence of sterile abscesses, from other centers, has ranged between 0% and 43% [1, 7, 39]. The yield of organisms by culture also directly depends on the prior use of antibiotics [11]. This could be one of the reasons for an increased incidence of sterile abscesses in group II of our study.

Infections with *P. aeruginosa and A. baumannii* are caused by contiguous extension to brain and meninges from an ear, mastoid, paranasal sinus surgery, or diagnostic procedures. In some patients, the involvement of the CNS is due to spread of the organism from infective endocarditis, pneumonia, or urinary tract infection [3]. These organisms have emerged as important and highly resistant nosocomial pathogens in recent years.

Brain abscess due to unusual Gram-negative bacilli was observed in the study group II only, probably due to the improved microbiological diagnostic techniques and automated identification systems used.

(i) *Salmonella typhi* is a rare pathogen in brain abscess and probably spreads to the brain hematogenously [40].

(ii) *B. pseudomallei* (agent of Melioidosis): brain abscess is a complication of a neurological infection [11]. Isolation of *B. pseudomallei* from the specimen remains the "gold standard" in diagnosis. The microbiologist should be well versed with the colony morphology and the antibiogram of these important yet not very fastidious organisms. Ceftazidime remains the drug of choice in the early phase of treatment, followed by a prolonged therapy with cotrimoxazole [14].

Anaerobic brain abscesses: the earlier studies on brain abscesses from India were specifically on anaerobic infections [41–43]. The obligate anaerobes are significant pathogens of a brain abscess and often occur as mixed infection either with another anaerobe or a facultative aerobe, as seen in our group. They are generally associated with otogenic and odontogenic infections.

Anaerobes are highly susceptible to metronidazole [41]. Subsequent to its addition to the antibiotic treatment regimen of a brain abscess and widespread use, the incidence of anaerobic infections has declined [1, 12, 41]. Attention to proper anaerobic isolation techniques is essential for a good recovery [11, 13] as is evidenced in the increased yield of anaerobes in our study group II (Table 2(c)). Since conventional anaerobic cultures require 2-3 days of incubation, it is essential to use alternative techniques for their early detection [44, 45]. Gas liquid chromatography (GLC) is a routine method used in the identification and differentiation of anaerobes from aerobes in clinical samples on the basis of the presence of volatile and nonvolatile fatty acids on the chromatogram. Computer-aided GLC is commercially available and is a means of rapid microbial identification [44, 45]. However, the equipment and cost are the limiting factors for a routine use of GLC in resource-restricted conditions.

Tuberculous brain abscess results when *mycobacteria* gain entry to the brain parenchyma, by a hematogenous route from a remote site, usually the lungs, unlike TB Meningitis, which occurs via lymphatic spread from cervical lymph nodes. The TB bacilli are immobilized in end arteries, which lead to formation of submeningeal tuberculous foci and either result in a tuberculoma or undergo a central caseation and liquefaction to form an abscess [46–49]. This phenomenon is very rare and commonly occurs in patients with cell-mediated immunity and is mostly focal and usually secondary to a primary focus in the lungs. Histologically and clinically, these abscesses are devoid of a granulomatous reaction, similar to pyogenic abscesses [19].

For appropriate therapy and clinical management, a TB abscess must be differentiated from a tuberculoma. The criteria for the diagnosis of a TB brain abscess, laid down by Whitener in 1978, should be fulfilled in the diagnosis of a TB brain abscess [50]. These include (i) evidence of a true abscess formation within the brain, as confirmed during surgery, (ii) histological proof of the presence of inflammatory cells in the abscess wall (histologically, the abscess walls are usually devoid of epitheloid and giant cells, unlike in a tuberculoma [13], and (iii) demonstration of AFB and isolation of *M.tb* from the abscess pus. Though not included in this data, we had reported a case of calvarial tuberculous osteomyelitic abscess, which was successfully managed [51].

Nocardial brain abscesses are rare and account for about 1-2% of all cerebral abscesses [52–54]. The entity is being increasingly reported in the present era of transplant surgeries and immunosuppressive therapies. The infection may occur as an isolated lesion or as a part of a disseminated infection of a pulmonary or a cutaneous infection [6, 11, 16, 34, 52, 53, 55]. Almost all the patients with *Nocardial* brain abscesses have a defective cell-mediated immunity [11]. An early detection and treatment are very important since the mortality is three times higher than that of other bacterial brain abscesses. Though *N. asteroides* and *N. brasiliensis* are the common species, other species are also being isolated from brain abscess [52]. The diagnosis of *Nocardial* brain abscess requires a high index of clinical suspicion, with an attempt for an early tissue and microbiological diagnosis [34].

Invasive fungal infections remain a life-threatening complication in children with hematological malignancies. The brain is a common site of haematogenously disseminated fungal infections from an extracranial focus [56]. Cerebral

aspergillosis occurs in about 10 to 20% of all cases of invasive aspergillosis and has a very poor prognosis. The outcome depends on the early recognition of the causative organism and prompt initiation of antifungal treatment. Hence, every attempt should be made to detect fungal filaments in the brain abscess material submitted [11].

9. Alternative and Advanced Techniques to Improve Detection of the Infecting Pathogen(s)

Microscopy-positive but culture-negative abscesses, especially the bacterial, are not unusual and pose a challenge to the microbiologist(s). The organisms, often the nutritionally demanding *Streptococcus* species and the anaerobic cocci, generally will not be recovered on routine culture media. Also cultures will be negative when the number of organisms in the abscess pus is less. Such specimen should ideally be processed for the etiologic agent by more sensitive assays. At present, these highly sensitive and sophisticated assays are being increasingly used in advanced centers for research purposes.

(1) Broad range real-time polymerase chain reaction (RT PCR) using 16srRNA or DNA will help amplify and detect the probable bacterial nucleic acid, as shown by several studies [57–62]. A subsequent sequencing of the amplified product is performed to further improve the specificity. With the recent advances, the molecular methods are gradually making inroads into routine diagnostic laboratories and in the coming years may become important primary tools in the early detection of the etiologic agent. However, the specificity and the cost of these assays are important limiting factors [63] in the underdeveloped and developing countries.

(2) *In vitro* nuclear magnetic resonance (NMR) used for detecting the specific spectral pattern of the amino acids and other volatile substances released by metabolic processes of the bacteria has been applied as a means of differentiating abscesses from other space-occupying lesions in the brain [64–66]. However, the need for NMR with very high Tesla and an expert analysis and interpretation of the spectral patterns are the limiting factors.

(3) Gas liquid chromatography (GLC) on the pus sample is also an alternative method, especially for the anaerobic abscesses.

10. Conclusions

As evidenced by our data, the microbiology of intracranial abscesses is complex. The detection and identification of the causative pathogen(s) are the cornerstones of diagnosis for an appropriate management and therapeutic optimization. The value of a promptly evaluated microscopy coupled with meticulous and elaborate culture methods of the abscess material cannot be under-estimated. The high yield of positive cultures will then enable the neurosurgeon and the infectious disease specialist to treat brain abscess more rationally and appropriately. Abscesses rarely arise *de novo* within the brain [4, 11]. There is almost always a primary lesion elsewhere in the body that must be sought assiduously, since failure to treat the primary lesion will result in relapse [11]. The need to sample the primary source of the brain abscess, especially lungs, when unusual pathogens are isolated from the brain abscess (even in cryptogenic abscesses), is emphasized through our series.

It is advisable for the neurosurgeon to co-ordinate closely with the microbiologist and ensure application of advanced microbiological and molecular techniques, to detect the new and emerging agents of intracranial abscesses.

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Review Article

Pathology of Acute Henipavirus Infection in Humans and Animals

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Zoonoses as causes of human infections have been increasingly reported, and many of these are viruses that cause central nervous system infections. This paper focuses on the henipaviruses (family Paramyxoviridae, genus *henipavirus*) that have recently emerged to cause severe encephalitis and systemic infection in humans and animals in the Asia-Pacific region. The pathological features in the human infections comprise vasculopathy (vasculitis, endothelial multinucleated syncytia, thrombosis, etc.) and parenchymal cell infection in the central nervous system, lung, kidney, and other major organs. Most animals naturally or experimentally infected show more or less similar features confirming the dual pathogenetic mechanism of vasculopathy-associated microinfarction and direct extravascular parenchymal cell infection as causes of tissue injury. The most promising animal models include the hamster, ferret, squirrel monkey, and African green monkey. With increasing evidence of infection in the natural hosts, the pteropid bats and, hence, probable future outbreaks in many more countries, a greater awareness of henipavirus infection in both humans and animals is imperative.

1. Zoonotic Viruses Associated with Viral Encephalitis

Numerous emerging infections are zoonoses of known or newly discovered viruses that have jumped the species barrier to infect humans. These include the human immunodeficiency virus (HIV), arboviruses, lyssavirus, henipaviruses, avian, and swine influenza viruses [1–8]. Many of these zoonotic viruses cause severe encephalitis associated with significant mortality and morbidity.

Since its origin has been traced to African nonhuman primates, HIV has become established in human populations [1]. The prevalence of HIV encephalitis is unknown; perhaps hundreds of thousands suffer from this condition since millions of HIV-infected patients still do not have adequate antiretroviral therapy. Among the arboviruses, Japanese encephalitis virus (JEV) infections, transmitted by mosquitoes from birds, is probably the most important, with more than 50,000 patients from the Indian subcontinent and southeast Asia [5]. West Nile virus, another known, similarly transmitted arbovirus, recently emerged to cause human neuroinvasive disease in North America, a region not previously known to be affected [9, 10].

Henipavirus genus, a recently established group of paramyxoviruses [11] comprising the Hendra virus (HEV) and Nipah virus (NIV), has emerged to cause severe encephalitis in humans and animals. There are several previous reviews on NiV or henipavirus infections [12–17], but the present one focuses on the epidemiology, clinical features, and comparative pathology in infected humans and animals and also includes some previously unpublished data.

HeV was first isolated after an outbreak in horses and 2 humans in the town of Hendra, QLD, Australia in 1994. Since then several other small outbreaks involving horses only, or horses and their carers, have been reported only in Australia and mainly in Queensland. Scores of horses and 7 humans (4 fatalities) have been infected so far [17–24]. NiV was named after the Nipah River village in Malaysia, very soon after the first known outbreak occurred mainly around pig farms from 1989 to 1999. Although a prevalence of 265 Malaysian cases of acute NiV encephalitis with 105 fatalities has been reported [25], the subsequent spread of the virus

to Singapore and its ability to cause mild infections [26] suggested that the total number infected was probably more than 350 cases [14]. After the outbreak was controlled in Malaysia and Singapore in1999, at the beginning of 2001, several recurrent NiV outbreaks were reported from Bangladesh and the adjacent Bengal area of India [27, 28] that have involved more than 120 people thus far.

2. Henipavirus Transmission

The natural host of henipaviruses is the fruit bat (*Pteropus* species or "flying foxes") [29–31], and bat-to-human transmission may be direct or indirect via intermediate hosts. The horse is the main if not the only intermediate host for HeV transmission [18, 23, 24]. Numerous other domestic animals and wildlife investigated were negative for naturally acquired HeV infection [21]. Contact with virus in horse oronasal secretions and urine appears to be the most likely route of transmission [32, 33]. Although person-to-person HeV transmission has not been reported, involvement of the lung and kidney in acute infection and presence of virus in nasopharyngeal secretions strongly suggest this possibility. The natural mode of bat-to-horse transmission remains unclear and unproven experimentally [32]. It was suggested that ingestion of feed or pasture contaminated by bat-derived foetal tissues or urine may be responsible.

In the Malaysia/Singapore outbreak, the pig was the main intermediate host and human transmission was strongly linked to close contact with pigs or fresh pig products [25, 34–37]. Massive culling of sick pigs and banning of exports stopped the epidemic [36, 38]. Similar to HeV, demonstration of virus in oropharyngeal/respiratory secretions suggests spread by either direct contact or aerosols [39, 40]. In contrast, absence of virus in pig urine could indicate that spread via urine may be inefficient. It was suggested that bat-to-pig transmission could have resulted from ingestion of half-eaten contaminated fruits dropped by bats near farms [29].

Person-to-person transmission in the Malaysian hospital setting is probably very low, but a nurse could have been infected from patients' tracheal secretions or urine [41–44]. There is no documentation of such transmission among and between farm workers and their families, but this remained a distinct possibility. In contrast, in the Bangladesh/India outbreaks, there was a high incidence of person-to-person transmission involving health care workers or other people [28, 45, 46]. No animals have been positively identified as intermediate hosts although there were associations with sick cows, pigs, and goats [45, 47] Bat-contaminated, date palm sap drunk raw as a local delicacy has been implicated in some cases of bat-to human transmissions in Bangladesh [48].

3. Clinical Aspects of Henipavirus Infection

The incubation period ranges from a few days to 2 weeks [19, 23, 24, 49, 50]. Milder symptoms include fever, headache, influenza-like illness, and drowsiness. Severe HeV infection, may present either as a neurological or a pulmonary syndrome, but since there have been very few patients, the clinical features were not well characterized. Neurological signs include confusion, motor deficits and seizures while the pulmonary syndrome presents with an influenza-like illness, hypoxaemia, and diffuse alveolar shadowing in chest X-Rays [23, 24]

Severe NiV encephalitic syndrome presents mainly with fever, headache, dizziness, vomiting, and reduced consciousness [50]. Clinical signs such as areflexia, hypotonia, abnormal pupillary and doll's eye reflex, tachycardia, hypertension, myoclonus, meningism, and convulsions were observed. A pulmonary syndrome has been described in some patients who present with cough, atypical pneumonia, and abnormal chest X-Ray findings [49–51]. Brain MR scans in acute henipavirus encephalitis show typical, disseminated, small discrete hyperintense lesions in both grey and white matter [23, 52, 53].

Specific antihenipavirus antibodies that can be detected in the serum and cerebrospinal fluid (CSF) in most patients are critical to diagnosis. More is known about seroconversion after NiV infection than HeV infection. In NiV infection, IgM seroconversion by about 2 weeks was 100% and persisted for more than 3 months. IgG seroconversion was 100% by about 3 weeks and may persist for several years [54, 55]. Specific neutralizing IgM or IgG antibodies have been reported in HeV-infected patients [19, 20, 24].

CSF examination showed elevated protein levels and/or white cell counts in more than 75% of NiV patients, but glucose levels were normal [50, 56]. Electroencephalography most commonly showed continuous, diffuse, symmetrical slowing with or without focal discharges in acute NiV encephalitis [57].

Mortality in HeV infection is about 50%, while in severe NiV infection it ranges from about 40% (Malaysia) to 70% (Bangladesh/India) [25, 27, 28]. In acute NiV encephalitis, brainstem involvement, presence of virus in the CSF, and diabetes mellitus are poor prognostic indicators [50, 58, 59]. The majority of Malaysian patients apparently recovered with no serious sequelae. However, henipavirus infection may be complicated by relapsing encephalitis after initial recovery. One case of relapsing HeV encephalitis and more than 20 cases of relapsing NiV encephalitis (probably <10% of survivors) have been reported thus far [20, 26]. The single case of relapsing HeV encephalitis occurred about 13 months after exposure, while an average of 8 months elapsed before relapsing NiV encephalitis occurred. Some cases of relapsing NiV encephalitis only had fever and headache during the acute phase and have also been called "late-onset" encephalitis. Clinical, radiological, and pathological findings suggest that relapsing NiV encephalitis is distinct from acute NiV encephalitis and that relapsing henipavirus encephalitis is the result of viral recrudescence [13, 26, 52, 60].

4. Pathology of Acute Henipavirus Infection in Humans

Although published data on HeV infection consists of a single case and most of the information on human henipavirus infection is derived from NiV studies, we believe both viruses cause essentially the same pathology. Acute infection

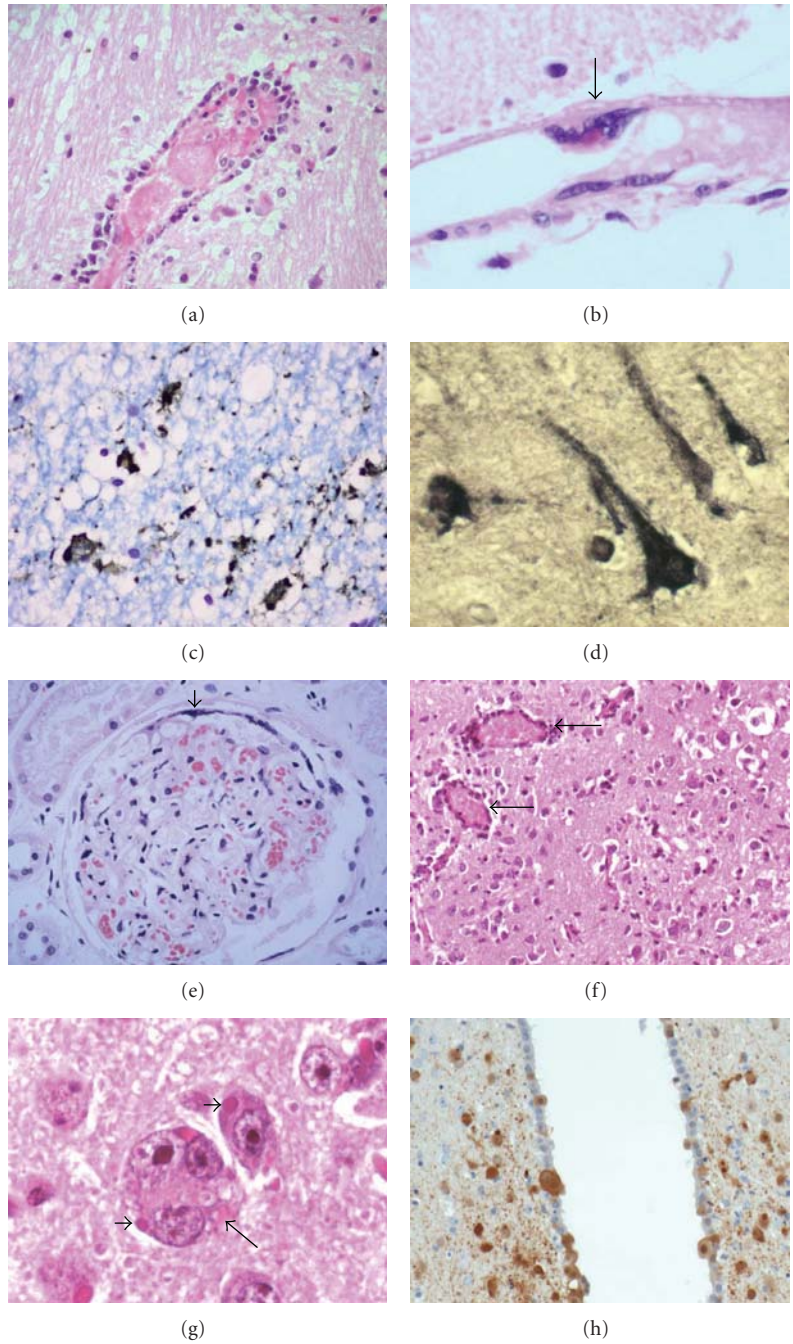

Figure 1: Pathology of human and hamster henipavirus infection. (a) Vasculitis and associated intravascular thrombosis in human brain. (b) In an uninflamed meningeal vessel, a multinucleated giant cell (arrow) with viral inclusion arises from the endothelial surface. (c) Neuronal viral antigens in human Nipah infection. (d) Neuronal viral RNA in human Hendra infection. (e) Glomerulus in human Nipah infection with thrombosis, necrosis, and peripheral multinucleated giant cell formation (arrowhead). (f) Mild vasculitis (arrows) and encephalitis in Nipah-infected hamster brain. (g) Viral inclusions in neurons (arrowheads) and the rare neuronal syncytia (arrow) in Nipah-infected hamster brain. (h) *Nipah* viral antigens in neurons and ependymal cells in infected hamster. (h and e) stains (a, b, e, f, g), immunoperoxidase stains (c, h), in situ hybridisation (d). Magnification, objective ×20 (a, c, f, h), ×40 (b, d, e, g).

is characterized by disseminated small vessel vasculopathy comprising true vasculitis, endothelial ulceration, and intramural necrosis in the central nervous system (CNS), lung, kidney, and many other major organs (Figure 1) [60, 61]. Occasionally, endothelial multinucleated giant cells or syncytia may be detected (Figure 1(b)). Vascular occlusion by vasculitis-induced thrombosis (Figure 1(a)) and perivascular haemorrhage were observed. Viral antigens, RNA, and nucleocapsids could be detected in vascular endothelium, multinucleated giant cells, and smooth muscle [61, 62].

In NiV infection, CNS vasculopathy was most severe compared to other organs. Vasculopathy was often associated

with discrete necrotic or more subtle vacuolar plaque-like lesions that corresponded with lesions seen in the MR scans. These lesions were characterized by necrosis, oedema, and inflammation, and often viral antigens (Figure 1(c)) and RNA (Figure 1(d)) were demonstrable in adjacent neurons [60]. Hence, it is believed that both microinfarction and neuronal infection give rise to necrotic plaques. In some cases, focal neuronophagia, microglial nodule formation, clusters of foamy macrophages, perivascular cuffing, and meningitis can be found. A more extensive review of the CNS pathology has been published elsewhere [13]. In the lung, kidney (Figure 1(e)), lymphoid organs, and so forth, vasculopathy, parenchymal inflammation, and necrosis with occasional multinucleated giant cells were also observed [60, 61].

5. Pathology of Acute Henipavirus Infection in Animals

Consistent with *in vitro* experiments that showed extensive infectivity of henipaviruses in different cell lines [63], natural or *in vivo* experimental infections on a variety of mammalian species have been reported. The table summarises these findings and is organized on the assumption that henipaviruses as a group probably causes similar pathology in the same animal species. We are aware there may yet be differences between HeV and NiV infections in the same animal, but to date there is no published study that directly compares these viruses under identical experimental conditions.

Animals naturally infected by henipaviruses and whose tissues have been examined for pathological changes are few and include the dog, cat, horse, and pig [32, 40, 66, 84]. Hooper et al. described pulmonary inflammation and glomerular and tubular necrosis associated with syncytia formation in NiV-infected dogs [67]. We examined two naturally infected dogs and found pulmonary vasculitis (Figure 2(a)), alveolar oedema, and inflammation (unpublished data). In the kidney, many glomeruli and adjacent tubules were thrombosed or necrotic with varying degrees of inflammation (Figure 2(b)). Viral antigens and RNA were demonstrated (Figure 2(c)). Serological studies confirmed that the dog is susceptible to NiV infection [71], but susceptibility to HeV was inconclusive [21, 68]. Nonsuppurative meningitis, cerebral ischaemia and vasculopathy have been described, but there is no published data on direct neuronal infection [67].

The cat is very susceptible to henipavirus infection under natural or experimental conditions. Vasculopathy consisting of vasculitis, endothelial syncytia, and viral immunolocalisation in endothelium and vascular smooth muscle was observed in many organs, except perhaps in the brain parenchyma, but meninges were involved (Table 1). There is severe pulmonary inflammation, and bronchial epithelium involvement may be prominent [40, 66, 67, 69]. Lymphoid tissues, such as the spleen, lymph nodes, thymus, and Peyer's patches, and kidney parenchymal tissues including glomeruli were often involved.

The horse as the intermediate host of HeV develops both a pulmonary and an encephalitic syndrome [18, 33], the latter being recognized only more recently. As in other infected animals, systemic vasculopathy is a prominent feature in the lungs, CNS, kidney, and other organs (Table 1). Apart from vasculopathy, observed encephalitis, necrosis and neuronal changes in the CNS suggest direct neuronal infection, but surprisingly so far there are no published reports to confirm this [67].

Naturally NiV-infected pigs develop a distinctive clinical syndrome called "porcine respiratory and encephalitis syndrome" or "barking pig syndrome" [85]. As the latter name suggests, pigs can develope a characteristic loud barking cough, which differs from other known porcine respiratory diseases. Respiratory distress was also observed in pigs experimentally infected with henipaviruses [39, 81]. Neurological signs included paralysis and abnormal movement and gait. Many pigs however may remain asymptomatic or, having developed clinical signs and symptoms, recover to a large extent [85].

In studies of both natural and experimental pig infections that we (unpublished data) and others have done, the most severe pathology appears to be found in the respiratory system [39, 40, 81–83]. There was evidence of tracheitis, bronchial inflammation, and pneumonia. Numerous macrophages, neutrophils, and multinucleated cells can be found within alveoli (Figures 2(d) and 2(e)) and bronchioles. Epithelial syncytia arising from the bronchial epithelium are prominent, and viral antigens and RNA (unpublished data) could be demonstrated (Figures 2(f) and 2(h)). Vasculitis and multinucleated syncytial cells were seen in small blood vessels (Figure 2(i)). Meningitis was characterised by vasculitis, inflammation, and viral antigens localised to the arachnoid membrane [40, 67]. Overall, encephalitis was thought to be rare, but neuronal and peripheral nerve infections have been demonstrated [39, 82]. Peripheral nerves may play a role in viral transmission into the CNS, a phenomenon suggested so far only in the pig.

Several other animals that have been experimentally infected successfully include the guinea pig, hamster, ferret, nonhuman primates (squirrel monkey and African green monkey), and chick embryo (Table 1). The infected guinea pig shows extensive vasculopathy (Table 1) in the urinary bladder, female reproductive tract, lymphoid organs, gastrointestinal tract, brain, and so forth. [64, 65, 74, 75, 84]. Notably, although pulmonary vasculopathy was described [67], the lung generally showed mild inflammation. Viral antigens and inclusions could be localised to neurons [74], but higher viral doses may be needed to produce encephalitis and/or neuronal infection [75]. Hamster tissues infected by henipaviruses generally showed systemic vasculopathy and parenchymal lesions in most major CNS and non-CNS organs examined (Table 1) [76, 77]. In the CNS, there was encephalitis, and there were viral inclusions, antigens and RNA in the neurons (Figures 1(f) and 1(h)). Very rarely, neuronal syncytia were observed (Figure 1(g)) (unpublished data). In addition to vasculopathy, pneumonia, glomerulitis and tubular lesions have been described. The squirrel monkey and African green monkey are susceptible by henipaviruses, and results suggest that they are good nonhuman primate animal models. As in the human infection, systemic vasculopathy and involvement of a broad range of organs

Table 1: Summary of animal susceptibility to henipavirus infection and range of pathologies reported in the literature.

Animal	Susceptibility to Hendra virus		Susceptibility to Nipah virus		CNS pathology of henipavirus infection		Non-CNS pathology of henipavirus infection		Remarks	Refs
	Natural infection	Experimental infection	Natural infection	Experimental infection	Vasculopathy***	Parenchymal lesions	Vasculopathy	Parenchymal lesions		
Bat	Yes	Yes	Yes	Yes	Yes Mainly meninges	NR	Yes Gastrointestinal tract, kidney, spleen, placenta, lung	Yes Kidney, heart, liver, salivary gland, testis, lung, trigeminal ganglion, intestine, urinary bladder, prostate	Neuronal infection not reported so far	[29, 31, 32, 64, 65]
Cat	NR*	Yes	Yes	Yes	Yes Meninges mainly	Yes Mainly meningitis	Yes Lung, gastrointestinal tract, kidney, urinary bladder, heart, liver, lymphoid organs	Yes Lung, urinary bladder, kidney, lymphoid organs, gastrointestinal tract	Bronchial epithelium infection prominent. Encephalitis /neuronal infection rare	[21, 40, 66–69]
Chicken embryo/ adult	NR	NA**	NA	Yes	Yes	Yes	Yes Heart, lung, liver, kidney, spleen, proventriculus, skin, peripheral ganglion, yolk sac	Yes Heart, lung, kidney, spleen, skin, feather, allantochorion, proventriculus, peripheral ganglion		[21, 70]

Table 1: Continued.

Animal	Susceptibility to Hendra virus		Susceptibility to Nipah virus		CNS pathology of henipavirus infection		Non-CNS pathology of henipavirus infection		Remarks	Refs
	Natural infection	Experimental infection	Natural infection	Experimental infection	Vasculopathy***	Parenchymal lesions	Vasculopathy	Parenchymal lesions		
Dog	NR	NR	Yes	NA	Yes	Yes	Yes Lung, kidney, liver	Yes Lung, kidney		[21, 67, 68, 71], authors' unpublished data
Ferret	NA	NA	NA	Yes	Yes	Yes	Yes Lung, kidney	Yes Lung, kidney, lymphoid organs, urinary bladder, adrenal cortex, fallopian tube, thyroid		[72, 73]
Guinea pig	NA	Yes	NA	Yes	Yes	Yes	Yes Lymphoid organs, urinary bladder, female genital tract, gastrointestinal tract, skeletal muscles, placenta, adrenal gland, thymus, thyroid, heart, lung, kidney	Yes Lymphoid organs, urinary bladder, female genital tract, lung, kidney, gastrointestinal tract, adrenal gland, thymus, thyroid, heart	Encephalitis and neuronal infection more prominent with higher Hendra virus doses. Lung mainly mild inflammation	[64–66, 74–76]
Hamster	NA	Yes	NA	Yes	Yes	Yes	Yes Lung, kidney, liver, heart	Yes Lung, kidney, spleen, heart		[76, 77]
Horse	Yes	Yes	Yes	NA	Yes	Yes	Yes Lung, lymphoid organs, kidney, heart, gastrointestinal tract, urinary bladder, skeletal muscle	Yes Lung, kidney, lymphoid organs, gastrointestinal tract	Neuronal infection not reported so far	[18, 32, 66, 67]
Mouse/ Rat	NR	NA	NA	No	NA	NA	NA	NA		[21, 76]

Table 1: Continued.

Animal	Susceptibility to Hendra virus		Susceptibility to Nipah virus		CNS pathology of henipavirus infection		Non-CNS pathology of henipavirus infection		Remarks	Refs
	Natural infection	Experimental infection	Natural infection	Experimental infection	Vasculopathy***	Parenchymal lesions	Vasculopathy	Parenchymal lesions		
Non human primates (Squirrel monkey, African green monkey)	NA	Yes	NA	Yes	Yes	Yes	Yes Lung, gastrointestinal tract, tongue, salivary gland, larynx, heart, gall bladder, sex organs, endocrine glands, skeletal muscle	Yes Lung, kidney, spleen, urinary bladder	The African green monkey may be more susceptible to infection than squirrel monkey	[78–80]
Pig	NR	Yes	Yes	Yes	Yes Meninges mainly	Yes Mainly meningitis	Yes Lung, nasal turbinate, heart, kidney, lymphoid organs, gastrointestinal tract	Yes Lung, kidney, lymphoid organs, larynx, peripheral nerves, tonsil, nasal turbinate	Bronchial epithelium infection very prominent. Encephalitis /neuronal infection rare	[16, 21, 40, 67, 81–83], authors' unpublished data

*NR= not reported; studies done.
**NA= not available; no studies done.
***Vasculopathy and parenchymal lesions, respectively, includes morphological changes and/or immunolocalisation of viral antigens.

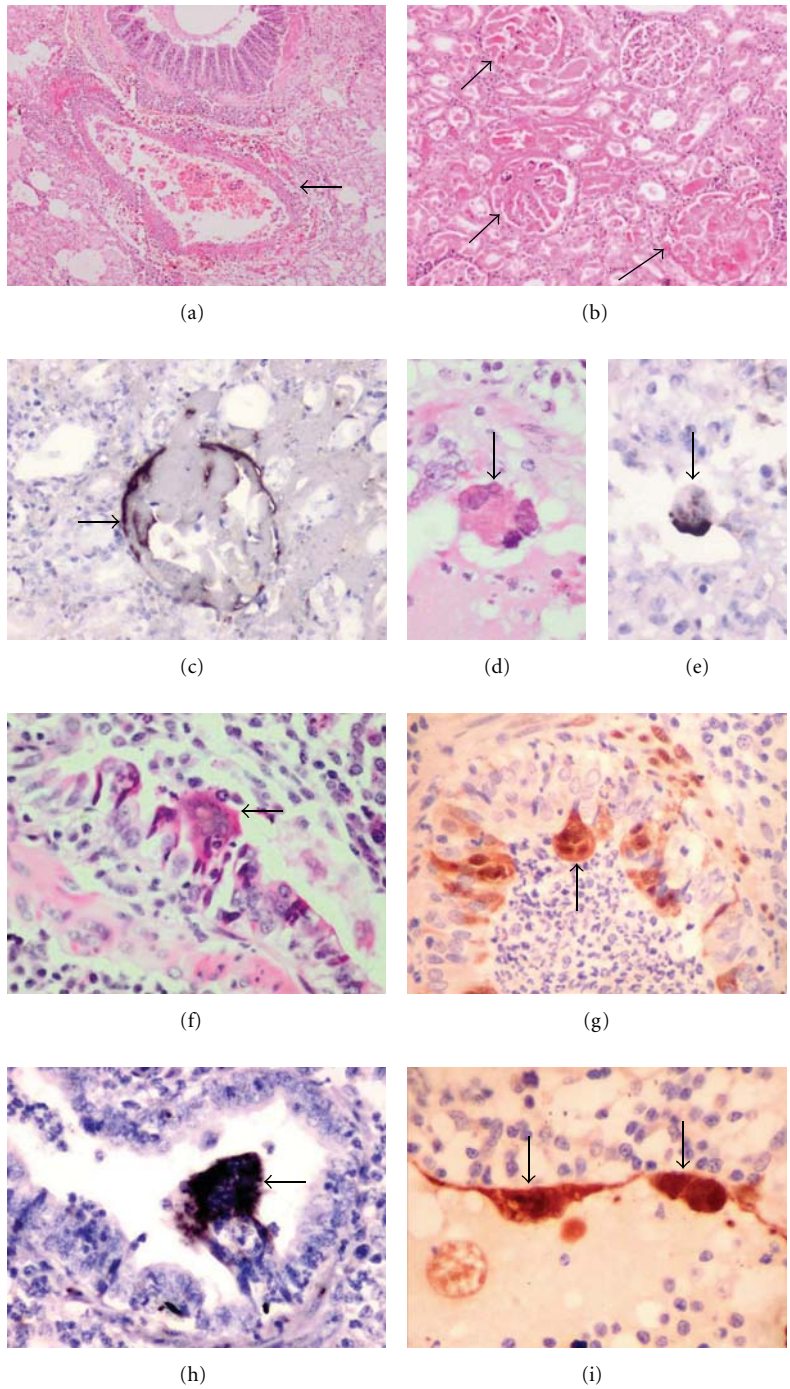

Figure 2: Pathology of dog and pig henipavirus infection. (a) Pulmonary vasculitis (arrow) and oedema in the Nipah-infected dog lung. (b) Glomerular (arrow) and tubular necrosis in the dog kidney. (c) Nipah viral RNA in dog glomerulus (arrow). Intra-alveolar multinucleated giant cell containing Nipah viral inclusions (d) (arrow) and viral RNA (e) (arrow). Bronchiolar syncytia (f) (arrow), viral antigens (g) (arrow) and RNA (h) (arrow) in Nipah-infected pig lung. Endothelial giant cell in pig pulmonary vessel (i) (arrows). (h and e) stains (a, b, d, f), immunoperoxidase stains (g, i), in situ hybridisation (c, e, h). Magnification, objective ×4 (a), ×10 (b), ×20 (c), ×40 (d-i).

were detected (Table 1) [78–80]. More detailed analysis of the pathological features in these models should enable the pathogenesis of henipavirus infection to be further investigated. Pathological data from the infected ferret shows systemic vasculopathy and parenchymal lesions in the CNS and non-CNS organs (Table 1) [72, 73]. The chick embryo also shows evidence of extensive CNS and non-CNS involvement suggesting that adult birds may also be susceptible to henipaviruses, but so far there is no data available [70].

As the natural reservoir host of henipaviruses, it is not surprising that experimentally infected bats did not develop severe disease nor severe pathological changes (Table 1)

[64, 65]. Interestingly, mouse and rat do not apparently develop clinical disease for reasons yet to be investigated [76].

In general, the pathology described in various animal species reflects the pathological features seen in the human disease, namely, extensive vasculopathy, parenchymal lesions in multiple organs, and evidence of viral infection. However, there may be some significant differences among animals. In the pig and cat, respiratory tract involvement, notably of the bronchial epithelium, stands out as a prominent feature. In contrast, the guinea pig shows mild lung parenchymal inflammation. Encephalitis and/or neuronal infection may be more subtle in the pig and cat in contrast to human infection.

The pathological findings in the respiratory tracts of the horse and pig, particularly the latter, are of course consistent with the postulated modes of viral transmission to humans via oropharyngeal/respiratory fluids and aerosols. Interestingly, negative virus isolation from pig urine suggests inefficient viral spread by this means [39, 81] though rare involvement of the glomerulus still suggests this possibility [40]. We were unable to demonstrate glomerular or tubular pathology in the 2 pigs that we have examined (unpublished data). Thus, respiratory tract secretions may be the main mode of pig-to-human NiV transmission. On the other hand, extensive kidney involvement in dogs and cats, implicated as minor intermediate hosts, may be via contaminated urine and in cats via respiratory secretions as well [25, 67, 71, 86].

If one considers as a prerequisite for a good animal model encephalitis and neuronal involvement in the CNS, in addition to systemic vasculopathy and severe inflammation in the lung, kidney, and other major organs, then perhaps the hamster, ferret, and monkey represent the best available small animal models of henipavirus infection. Although it is difficult to directly compare the relative susceptibility of these animals to henipaviruses as the viral sources and doses, inoculation routes, and animal and environmental characteristics may be different, perhaps among the nonhuman primates, the African green monkey could be more susceptible than the squirrel monkey. Nonetheless, all these models could be useful models for pathogenesis, therapeutic and vaccine studies as have already been done [77, 79, 87]. Overall, all the animal models confirm the dual pathogenetic mechanisms postulated for tissue injury in henipavirus infection, namely, vasculopathy-associated microinfarction and direct viral infection of extravascular parenchymal cells [61].

It is perhaps not surprising that henipaviruses cause similar infectious disease pathology in both humans and animals as it has now been shown that they share the same virus entry receptor. The main receptor has been identified as ephrin B2 [88, 89], and the alternative receptor is ephrin B3 [90]. These receptors are ubiquitous on plasma membranes of many mammalian cells, particularly in the blood vessels and CNS, thus accounting for the prominent clinic pathological features of vasculitis and CNS involvement.

The emergence of henipaviruses over a short period of a few years underscores the growing importance of this group of viruses as causative agents of previously unknown zoonoses. Because pteropid bats as natural hosts are found in many parts of the world, future henipavirus outbreaks should be anticipated [29, 30, 91–95].

6. Addendum

A very recent comparative study of NiV and HeV in the hamster model (Rockx et al. [96]) showed that while the type of pathological lesions was essentially similar between the two, there were differences in respiratory tract replication sites and the onset and severity of pathological lesions. HeV-induced lesions were found to appear earlier and were more severe.

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Research Article

Aspartoacylase Deficiency in the White Matter of Human Immunodeficiency Virus Encephalitis: Novel Mechanism in Axonal Damage

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Aspartoacylase/aminoacylase II (ASPA/ACY II) is mainly synthesized in oligodendrocytes to contribute in myelin synthesis. Although axonal damage is seen in the brain with human immunodeficiency virus encephalitis (HIVE), ASPA contribution in the pathology is not known. Immunostaining study showed that ASPA protein is reduced in the white matter of patients with HIVE compared to the control. Western blot study further confirmed ASPA deficiency in the HIVE brain compared to the control. This paper suggests that HIVE condition affects ASPA to contribute in myelin loss/axonal damage seen in the disease.

1. Introduction

Human aspartoacylase/aminoacylase II (ASPA/ACY II; EC no. 3.5.1.15) gene contains five introns and six exons [1, 2]. Normal level of its substrate, N-acetylaspartic acid (NAA/NA-Asp) is important for the maintenance of healthy neurons. Altered levels of the NAA contribute in disease pathophysiology by inducing oxidative stress and by suppressing potential antioxidants [3–6]. Abnormal level of this pathway contributes in various diseases including Canavan disease [1–4], type 2 diabetes [7], and Parkinson's disease [8]. Aspartoacylase is mainly synthesized in oligodendrocytes to contribute in myelin synthesis [1, 2].

Human immunodeficiency virus encephalitis (HIVE) is a demyelinating disease of the central nervous system, caused by the lethal virus [9–11]. Approximately 2.7 million new HIV-1 infections and 2.0 million deaths due to AIDS were reported in 2008 [12, 13]. In North America, the epidemic is expanding in the population among men who have sex with men [14, 15]. Brain regions affected in the disease include basal ganglia and deep white matter [16, 17], and these brain regions are also affected in the brain with Canavan disease [1, 2], therefore studying aspartoacylase level in the white matter of the patients with HIVE is important. Thus, the present study was aimed to understand ASPA level in the white matter of patients HIVE.

2. Materials and Methods

Six brain samples each from control and HIVE were used. While control brains showed no histologic abnormalities, HIVE brain showed leucoencephalopathy. All the procedures were performed under the regulations of institutional ethical committee and with the Helsinki Declaration of 1975, as revised in 2000 (World Medical Association Declaration of Helsinki 2000). To perform immunostaining, paraffin sections from three each of control and HIVE brain were deparaffinized in xylene, rehydrated in graded ethanol and incubated with ASPA antibody (Santacruz, CA) as followed earlier [18]. The slides were then washed in PBS and incubated with antirabbit conjugated Alexa-fluor 488 (Molecular probes, CA). Sections were photographed as described earlier [18].

To confirm the immunofluorescence findings, western blot was performed using three of each control and HIVE

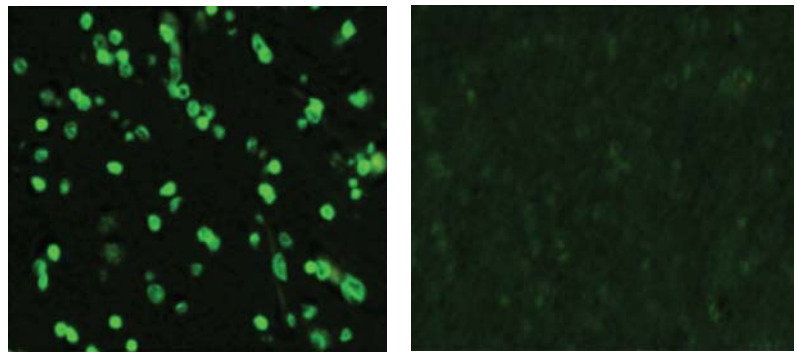

Figure 1: Immunofluorescence study of aspartoacylase in the brain of patients with HIVE. Aspartoacylase protein is reduced in the fore brain of HIVE patients compared to the control (magnification, 20x).

brain samples. Three brain samples of each control and HIVE were homogenized in lysis buffer and western blot was performed as followed earlier [19]. Fifteen-microgram protein from control and HIVE brains was loaded onto a 12% gel and the protein transferred nitrocellulose membrane was blocked with 5% blocking buffer. Then the membrane was incubated with ASPA antibody (Santa Cruz, CA) 1 : 200 dilution. After washing with PBS, membrane was incubated with anti-rabbit HRP antibody (Invitrogen, CA). The protein band was detected using supersignal west pico chemiluminescent substrate (Fisher scientific, IL) and photographed as described earlier [19]. Density of the bands was also measured as described earlier [19]. Statistical analysis was performed using ANOVA. $P < 0.05$ was considered as significant.

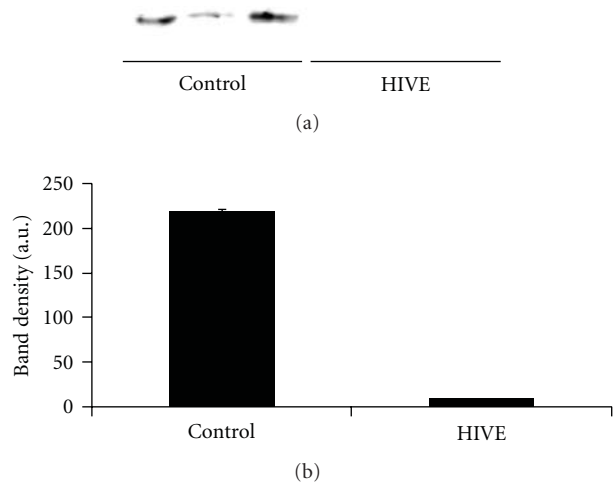

Figure 2: Western blot study of aspartoacylase protein in the brain of patients with HIVE. (a) Aspartoacylase protein was deficient in the brain of patients with HIVE compared to the control. (b) Density analysis of the band showed reduced amount of ASPA protein in the brain with HIVE compared to the control brain. Two-tailed P value was 0.02.

3. Results and Discussion

Immunostaining of the HIVE brain white matter showed reduced level of ASPA compared to the control (Figure 1). These fluorescent cells were colocalized with oligodendrocyte marker (Data not shown). Western blot study also further confirmed the reduced level of ASPA in HIVE brain compared to the control (Figure 2). Density analysis of the bands showed that two-tailed P value was 0.02.

HIV has been a devastating disease over decades and white matter degeneration is also reported [20, 21], however, ASPA contribution in the white matter degeneration is not known. ASPA is mainly synthesized in oligodendrocytes [1, 2] and a reduced level of ASPA impedes myelination and thus leads to axonal damage [1, 2, 22, 23].

Human immunodeficiency virus infection starts from periphery and subsequently enters the central nervous system but neurological symptoms occur years later. Axonal damage is reported in the brain with HIVE [24, 25]. Monogene alters other genes expression to contribute in disease pathophysiology [2]. HIV is capable of inserting with genomic DNA [26]. This insertion would impede function of other genes. Thus, deficiency of ASPA in the brain of patients with HIVE observed in the present study suggests that HIVE condition affects ASPA to contribute in myelin loss and axonal damage seen in the disease.

In conclusion, HIVE condition affects ASPA to contribute in axonal damage seen in the disease.

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Research Article

Role of Apoptosis in Rabies Viral Encephalitis: A Comparative Study in Mice, Canine, and Human Brain with a Review of Literature

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To evaluate the role of apoptosis in rabies encephalitis in humans and canines infected with wild-type street virus, in comparison with rodent model infected with street and laboratory passaged CVS strain, we studied postmortem brain tissue from nine humans, six canines infected with street rabies virus, and Swiss albino mice inoculated intramuscularly (IM) and intracerebrally (IC) with street and CVS strains. Encephalitis and high rabies antigen load were prominent in canine and human brains compared to rodents inoculated with street virus. Neuronal apoptosis was detectable only in sucking mice inoculated with CVS strain and minimal in street virus inoculated mice. In a time point study in suckling mice, DNA laddering was noted only terminally (7 days p.i.) following IC inoculation with CVS strain but not with street virus. In weanling and adult mice, apoptosis was restricted to inflammatory cells and absent in neurons similar to human and canine rabies-infected brains. Absence of neuronal apoptosis in wild-type rabies may facilitate intraneuronal survival and replication while apoptosis in inflammatory cells prevents elimination of the virus by abrogation of host inflammatory response.

1. Introduction

In Asia, canine rabies continues to be a serious public health problem causing large number of animal and human deaths. According to WHO estimates, 50,000 human deaths are reported worldwide every year [1], the majority from Asia and Africa, and 60% of them are from India alone [2]. Humans and canines acquire the infection by the introduction of virus-laden saliva following bite of a rabid animal [3] or rarely following mucosal exposure [4]. On gaining receptor-mediated entry into the neuron, the virus replicates and disseminates in the central nervous system by fast axonal transport along neuroanatomical connections. Clinically the disease manifests, either as furious (encephalitic) or paralytic (dumb) form of rabies, two-third of the victims suffering from furious form [5]. Unlike other viral encephalitides, examination of the brain reveals surprisingly minimal pathological alterations, in contrast to the dramatic clinical symptomatology.

Most studies on neuropathogenesis of rabies have employed animal models using laboratory-adapted viral strains. In natural hosts infected with virulent "street virus" strains, the pathogenetic mechanism mediating the disease is not well characterized. Experimental studies suggest dysregulation of neurotransmitters and ion channels or altered host immune responses as the cause of clinical symptomatology [6], but the cause of invariable fatality remains uncertain.

Recent reports suggest that viruses cause death of infected cells by apoptosis [7–10]. Apoptosis has also been implicated in pathogenesis of rabies, based on *in vitro* studies on neuronal cell lines as well as experimental studies in mice inoculated with laboratory-adapted virus strains [11, 12].

In natural hosts like bats (*Artibeus jamaicensis* bats) however, when infected with laboratory-adapted CVS-24 strain of rabies virus, Reid and Jackson failed to detect apoptosis in the neurons [13]. Yan et al. inoculating wild-type virus (silver-haired bat rabies virus) into experimental mice reported very few TUNEL-positive neurons, despite the presence of clinical signs of the disease, suggesting that apoptosis may not be an essential neuropathogenic mechanism [14]. In an HIV-1 positive individual bitten by a rabid dog, three months prior to clinical manifestation

of HIV-1, apoptosis was observed in neurons, macrophages, microglial cells, and oligodendrocytes in postmortem brain that was attributed solely to rabies virus infection and not considered to be induced by HIV-1 [15]. Jackson et al. evaluated the morphological features of neuronal apoptosis in postmortem brain tissue from 12 cases of human rabies collected from four different countries and suggested that neuronal apoptosis does not play an important role in human rabies encephalitis [16]. Apoptosis restricted to few inflammatory cells, but not neurons or glia in cases of canine rabies following natural infection with wild-type virus, was reported from our laboratory in a previous study [17].

To probe further the role of apoptosis in rabies infection, in the present communication, we compared the pathological features noted in natural hosts (human and canine) infected by street virus with the findings in susceptible swiss albino adult, weanling, and suckling mice, inoculated by intramuscular (IM) and intracerebral (IC) route with street virus (primary isolate from a canine brain) as well as laboratory-adapted CVS strains of rabies. Though no case of new born humans infected with rabies virus has been recorded and intracerebral inoculation does not reflect natural infection, these two events in mice are studied to enable comparison with other routes and ages. Histopathological features and neuroanatomical distribution of rabies viral antigen were examined in the human, canine subjects, and animal models. The phenomenon of apoptosis was evaluated by DNA fragmentation and TUNEL assay.

2. Materials and Methods

2.1. Experimental Mice. Swiss albino {suckling mice (two days old), weanling (21 days old), and adults (3 months)} obtained in batches from the same litter (both sexes) were sourced from the Central Animal Research Facility, National Institute of Mental Health and Neurosciences, Bangalore, South India. The animals were housed in spacious cages maintained at ambient temperature (24°C) with 12 hr light and dark cycles and access to pelleted food and water *ad libitum*. For experiments on suckling mice, litters of 10–15 animals were used. Weanling and adult test animals received 0.03 mL and suckling mice 0.01 mL of pretitered rabies virus (street virus and CVS strain-1000LD_{50} dilution), respectively. Suckling, weanling, and adult mice inoculated, respectively, with 0.01 mL and 0.03 mL of the vehicle, phosphate buffered saline served as negative controls. The study was approved by the Institutional Ethics Committee.

2.2. Virus Strain

(a) Stock of laboratory-adapted fixed rabies virus strain, CVS 11, was obtained as lyophilized mouse brain homogenate from the Central Research Institute, Kasauli, Himachal Pradesh, North India. CVS virus was prepared by passaging the rabies virus in suckling mouse brain as described previously [18].

(b) The brain tissue (hippocampus and cerebellum) from a confirmed case of furious rabies in a canine was homogenized in phosphate-buffered saline, and 20% suspension was aliquoted and stored at 70°C till use (primary isolate). This formed the source for "street virus" strain.

2.3. Experimental Design. Batches of adult, weanling and suckling mice were used for intramuscular (IM, $n = 3$) and intracerebral (IC, $n = 3$) inoculation with street and CVS strain of rabies virus, respectively. Intramuscular inoculation was administered in the right thigh. The intracerebral inoculation was at a point midway between the left ear and saggittal suture, into the parietal area (roughly corresponding to the sensory motor cortex). The animals in each group were monitored daily for 21 days for clinical features and the evolution of symptoms of rabies till they succumbed (Table 1). When the animals were found moribund and immobile with labored breathing indicating imminent death, they were euthanised by ether inhalation. Fresh brain tissues were collected from the frontal cortex for DNA laddering, a marker of apoptosis and immunoflourescence for rapid diagnosis. For histopathology, immunocytochemistry and TUNEL staining, 10% neutral-buffered formalin-fixed brains were used.

Human brain tissues ($n = 9$) collected at autopsy (following informed consent from close relatives), from confirmed cases of rabies and stored at the Human Brain Tissue Repository (Brain Bank), Department of Neuropathology, National Institute of Mental Health and Neurosciences, Bangalore were utilized for the study. They included both fresh (stored at −80°C) and formalin-fixed brain tissues from frontal, temporal, hippocampus, cerebellum, and medulla oblongata. Fresh brains from euthanised canines ($n = 6$) with a diagnosis of furious rabies were collected from the Department of Veterinary Pathology, University of Veterinary Sciences, Hebbal, Bangalore. Fresh canine brain samples from frontal, temporal cortex, and cerebellum were frozen at −80°C and the remaining were fixed in 10% buffered formalin for histological evaluation and immunohistochemistry to demonstrate viral antigen and TUNEL staining.

2.4. Histopathology. Standard haematoxylin-eosin staining on histological sections (five-micron thick), was examined for pathomorphological changes including microglial proliferation, perivascular inflammation, neuronophagia, and presence of intraneuronal eosinophillic Negri bodies in human, canine, and rodent brains in different anatomical areas were recorded. Cresyl-violet-stained adjacent sections were screened at high magnification (×40 objective) for nuclear pyknosis and apoptotic bodies in neurons, glia, and vascular endothelial cells.

2.5. Immunocytochemistry for the Detection of Rabies Viral Nucleocapsid Antigen. Representative sections from different neuroanatomical areas from human, canine, and rodent were immunostained with polyclonal antibody to rabies viral nucleocapsid (1 : 1500 dilution, specificity of the antibody was established by SDS-PAGE and western blot, which

Table 1: Comparison of the incubation period (IP) and duration of illness (DOI) with different routes of inoculation and strains of rabies virus.

Type of Virus and Route of inoculation		Incubation period (IP) and duration of illness (DOI)		
		Suckling mice (Age: 0–2 days)	Weanling mice (Age: 21 days)	Adult mice (Age: 3 months)
Street	Intracerebral			
	IP	8–10 days	6–8 days	6–8 days
	DOI	24 hrs	24 hrs	24 hrs
CVS	Intracerebral			
	IP	≤6 days	4–6 days	4–6 days
	DOI	48–72 hrs	48–72 hrs	48–72 hrs
Street	Intramuscular	Not done		
	IP		20–25 days	20–25 days
	DOI		24 hrs	24 hrs
CVS	Intramuscular	Not Done		
	IP		8–10 days	8–10 days
	DOI		48–72 hrs	48–72 hrs

Incubation period (IP): from bite to onset of clinical symptoms.
Duration of illness (DOI): from onset of clinical manifestation to death.

showed a single discrete band at the molecular weight 57 kDa, corresponding to rabies nucleoprotein) by standard indirect immunoperoxidase method. Appropriate negative controls (brain sections from an uninfected dog, mouse, and human brain and infected rabid dog brain treated identically but omitting the primary antibody) and positive controls (confirmed case of dog rabies, human rabies, and suckling mouse brain inoculated with rabies virus) were employed in each run to monitor the efficacy of the immunostaining process. The histopathological features and the extent of viral antigen distribution in different areas in the brain were graded semiquantitatively. Also representative sections from different neuroanatomical areas of human brain were immunostained with CD68 (Monoclonal, Biogenex, Calif, USA), marker for identifying macrophages/activated microglia.

2.6. DNA Laddering to Detect Apoptosis in the Brain. DNA was extracted from the fresh frozen rabies-virus-infected mouse brains (IC and IM inoculated suckling, weanling, and adult mice, three animals in each group), canine brain (hippocampus ($n = 6$)), human brain (frontal, temporal, hippocampus, and medulla oblongata ($n = 9$)) by the standard phenol-chloroform DNA extraction method. The extracted DNA was analyzed on agarose gel electrophoresis along with the molecular weight markers. Tissues from uninfected mice and canine brains and human brains with no evidence of rabies infection (brain collected from victims of road traffic accidents and stored) treated under similar conditions served as negative controls.

2.7. Temporal Evolution of Apoptosis in Suckling Mouse Brain Infected Intracerebrally with CVS Strain of Rabies Virus. Fresh brains from suckling mice infected intracerebraly with CVS strain of rabies virus were harvested at different time points—day 0, 24, 48, 72, 96, 120, 144 hrs, and DNA was extracted by standard phenol-chloroform extraction method and was analyzed by agarose gel electrophoresis.

2.8. In Situ TUNEL ASSAY for the Detection of Apop-tosis. To detect apoptotic nuclei, TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) staining was carried out using commercially available kit (ROCHE) as recommended by the manufacturer with appropriate positive and negative controls as previously described [17].

Double labeling for viral antigen and apoptosis could not be done for technical reasons, and; hence, immediate serial sections were stained and evaluated.

3. Results

The evolution of clinical signs (incubation period, IP) was earlier in mice infected with CVS strain compared to street virus, irrespective of the route of inoculation (Table 1). Mice inoculated with street virus had longer incubation period (8–10 days following IC inoculation and 20–25 days following IM inoculation), but the rate of disease progression was relatively rapid (24 hrs versus 48–72 hrs). Intramuscular inoculation with street virus caused paralysis of hind limbs followed by fore limbs. Following intracerebral inoculation of street virus, mice manifested ruffling of fur, hump back followed by limb paralysis within 6–8 days and survived for 48–72 hrs after the onset of symptoms.

Inoculation (IC or IM) with CVS strain of rabies virus produced similar symptomatology as with street virus, but of shorter incubation period, (8–10 days with IM and 4–6 days with IC route).

Neuropathological changes (perivascular inflammation and microglial reaction) in natural hosts (human, canine)

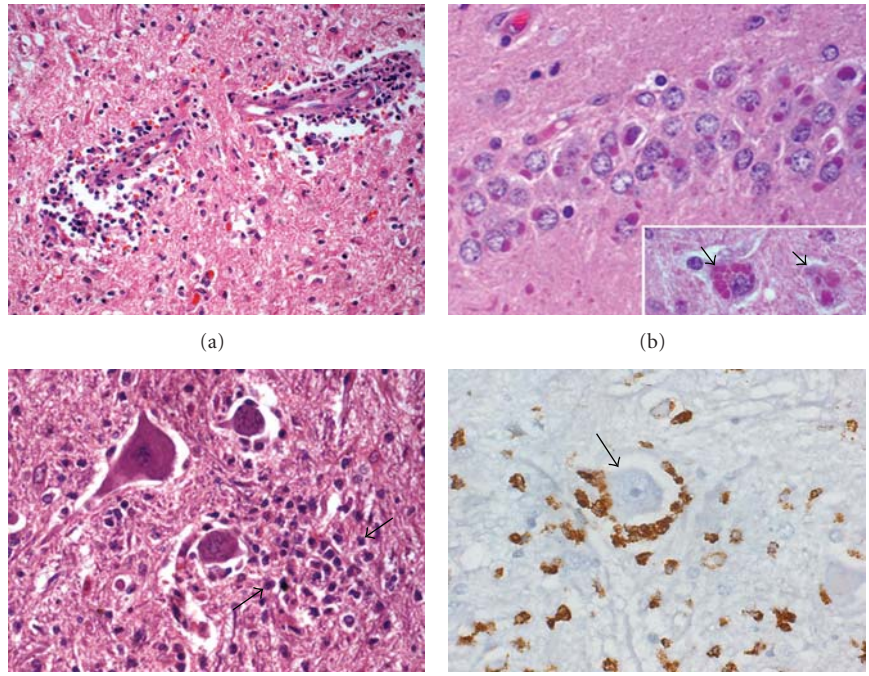

Figure 1: Human rabies viral encephalitis, 15 years/male, incubation period: 6 mon. Section from medulla oblongata showing dense perivascular cuffing of lymphomononuclear cells (a). Multiple Negri bodies are seen within granule neurons of hippocampal dentate gyrus (b). Inset shows multiple Negri bodies in hippocampal pyramidal neurons (b, inset, arrow). Anterior horn cells in the cervical segment of spinal cord are surrounded by microglial cells (c) Immunoreactive to CD68 (arrow, d). Note prominent nucleolus in the neuron reflecting viability. (a): HE ×120; (b): HE ×360; (b inset): HE ×360; C: HE ×300; D: Immunoperoxidase, CD68 ×300.

were more extensive than those in rodent brains (Figures 1(a)–1(c)). Variable degree of Negri body formation was seen depending on the incubation period (Figure 1(d)). In mice, perivascular inflammation, microglial nodule formation and negri bodies were more frequent in street-virus-infected mice, irrespective of route of inoculation. In CVS inoculated mice (IC & IM), perivascular inflammation and microglial reaction were mild while vacuolation of the neuronal soma was a striking feature. Negri bodies were undetectable. Suckling mice showed the least degree of inflammation.

Distinct differences were evident in the pattern and topography and the pattern of rabies viral antigen distribution in mice inoculated with street virus versus CVS strain (Table 2). In general, following street virus inoculation, widespread distribution of rabies viral antigen with a caudocranial gradient was observed by immunostaining. The highest concentration of antigen was seen in hippocampus (Figure 3(f)), thalamus, hypothalamus, amygdala, olfactory cortex, and cingulate gyrus while frontoparietal motor area as well as basal ganglia showed moderate number of infected neurons. As predictable, the quantum of viral antigen was considerably more following intracerebral route of inoculation. Within neurons, the viral antigen was seen aggregated into multiple globular masses (Figure 3(h)) with extensive dendritic spread (+++). In contrast, following inoculation with CVS strain (IM or IC) of rabies virus, weanling and adult mice showed diffuse cytoplasmic labeling with minimal dendritic spread (+) and extensive neuronal vacuolation. Neuroanatomically, the viral antigen was found mostly localized to specific sites like the ventral group of thalamic nuclei, hypothalamus, hippocampus (Figures 3(a) and 3(d)), and brainstem.

Topographical distribution of rabies viral antigen in the brain of naturally infected dogs and humans (street virus, peripherally inoculated by the animal bite) was diffuse and intraneuronal in almost all the anatomical areas (Figures 4(a) and 4(d)). Among the supratentorial structures, there was striking involvement of the lateral and ventral group of thalamic nuclei and the basal ganglia in addition to the limbic structures. Overall the highest antigen load was noted in cerebral hemispheres and along the brain stem.

High load of viral antigen irrespective of incubation period was noted in large neurons of reticular formation, vagal and hypoglossal nuclei. The pattern and morphology of antigen deposits were similar to that of mouse inoculated with street virus, forming multiple Negri bodies. In addition to considerable viral load in the neurons in natural hosts (humans and canines), antigen was also found in the oligodendrocytes and long cytoplasmic processes of the fibrous astrocytes in many areas, but significantly less in mice inoculated with two different strains of rabies virus and two different routes of inoculation suggesting variable permissiveness of neurons and glia in different species.

Table 2: Pathological features in mouse brain following inoculation with different strains of rabies virus with different routes of inoculation.

Virus type and route of inoculation		Histological features			Rabies viral antigen	Pattern of deposition
		PVI	Microglial response	Negri bodies		
CVS intracerebral	Suckling mice (Age: 0–2 days)	0–+	0–+	—	Widespread but specific localization to ventral thalamus, hypothalamus, hippocampus, limbic structures- cingulate, amygdala, and brain stem	Diffuse labeling, cytoplasmic vacuolation, minimal dendritic spread No Negri bodies
	Weanling mice (Age: 21 days)	+	+	—		Same
	Adult mice (Age: 3 months)	+	+	—		Same
CVS intramuscular	Suckling mice (Age: 0–2 days)	Not done	ND	ND	ND	ND
	Weanling mice (Age: 21 days)	+	+	—	Specific localization to ventral thalamus, hypothalamus, hippocampus, brain stem	Diffuse cytoplasmic labeling, extensive vacuolation, minimal dendritic spread No Negri bodies
	Adult mice (Age: 3 months)	+	+	—	Same	Same
Street intracerebral	Suckling mice (Age: 0–2 days)	++	++	+	More widespread	Multiple Negri body like intraneuronal aggregates, dendritic spread ++
	Weanling mice (Age: 21 days)	++	++	+	High density in thalamus, hypothalamus, hippocampus and limbic areas, brain stem, moderate in cortex, basal ganglia	Same
	Adult mice (Age: 3 months)	++	++	+		Same
Street intramuscular	Suckling mice (Age: 0–2 days)	Not done	NA	NA	NA	NA
	Weanling mice (Age: 21 days)	++	++	+	Widespread, caudocranial gradient	Intraneuronal aggregates, dendritic spread ++
	Adult mice (Age: 3 months)	++	++	+	Same	Same

Abbreviations used: PVI: perivascular inflammation, ND: not done, NA: not available.

Detailed neuroanatomical distribution of the viral antigen and immunophenotyping of the inflammatory cells will be presented separately.

Phenotypic expression of apoptosis with cresyl violet staining was evident only in CVS-virus-inoculated suckling mice but not in weanling or adult mice nor in street-virus-inoculated mice. Evidence of neuronal apoptosis was found in Ammon's horn neurons of hippocampus, as well as in the thalamus, hypothalamus, and cerebral cortex. Natural hosts (Human and Canine) did not demonstrate apoptosis in frontal, temporal, hippocampal regions, or in medulla oblongata.

DNA fragmentation (180–200 bp) and laddering confirmed the presence of apoptosis in suckling mouse brains infected with CVS strain of rabies virus. In the street-virus-infected mice, and sham-infected animals as well as in human and canine brains, no apoptosis was demonstrable (Figures 2(a), 2(b), and 2(c)).

Temporal evolution of apoptosis was noted in suckling mouse brain infected with CVS strain of rabies virus but not street virus. In a time point study, DNA laddering indicative of apoptosis was observed in CVS-strain-infected mouse brains only terminally in the disease process on the 6th day (144 hrs) postinoculation, while the brain at earlier time

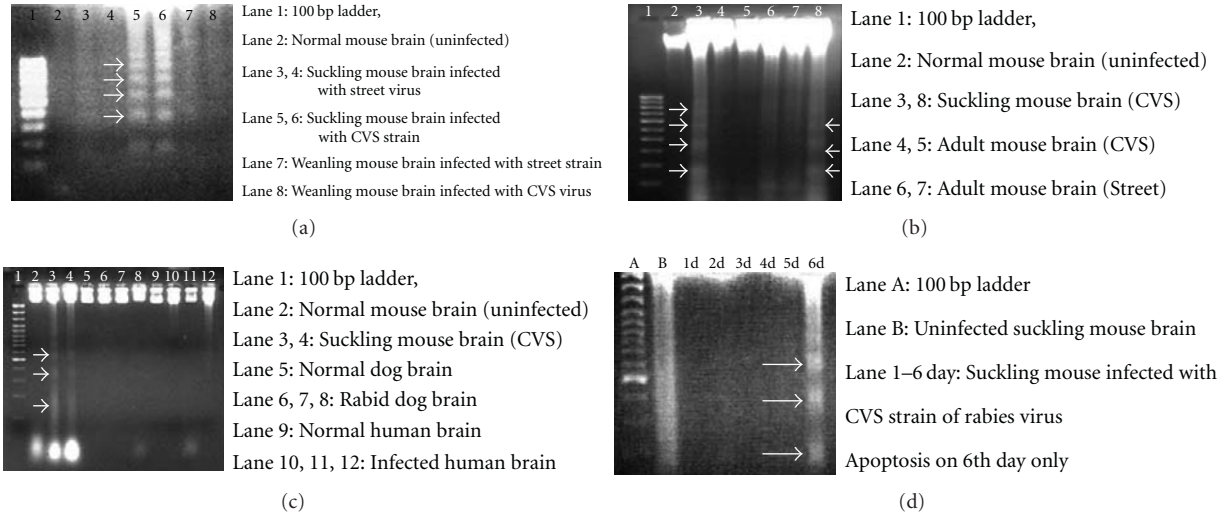

Figure 2: DNA laddering in Mouse brain. (a) DNA laddering (arrows) is seen only in suckling mouse brain infected with CVS strain of rabies virus (lanes 5 & 6). No laddering seen in street-virus-infected suckling mouse brain (lanes 3 & 4) and weanling adult mouse brain infected with street (lane 7) and CVS strain (lane 8) of rabies virus. (b) DNA laddering (arrows) in suckling mouse brain infected with CVS strain of rabies virus (lanes 3 & 8) and absence of laddering in street-virus-infected adult mouse brain infected with CVS and street strain of rabies virus. (c) DNA laddering (arrows) in suckling mouse brain infected with CVS strain of rabies virus and absence of laddering in street-virus-infected dog brain and human brain. (d) DNA laddering (arrows) in suckling mouse brain infected with CVS strain of rabies virus-time point study. DNA laddering exhibited only on the 6th day P.I.

points (0, 24, 48, 72, 96, 120 hrs) and uninfected mouse brain did not show a similar phenomenon, reflecting that apoptotic fragmentation of nuclear DNA was a terminal event (Figure 2(d)).

Terminal deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) positive apoptotic cells were detected widely distributed in suckling mice brain inoculated with CVS strain of rabies virus only and not in street-virus-inoculated ones or in naturally infected human and canine brain (Figure 3(b)). TUNEL-positive neurons were seen in all layers of the cerebral cortex, cingulate gyrus, Ammon's horn in hippocampus, and neurons in the thalamic, hypothalamic regions. In addition to neurons, the inflammatory cells lining the meninges, ependymal cells lining the ventricle, cells of the choroids plexus, and occasional glial and microglial cells in the cortex and white matter also showed labeling (Figure 3(c)). However, the endothelial cells lining the blood vessels were negative. Street-virus-infected suckling mice revealed TUNEL labeling of occasional microglial cells in the cerebral cortex and brain stem.

In the weanling and adult mice inoculated with CVS or street strain of rabies virus (IC and IM), neuronal cells failed to show apoptotic TUNEL immune labeled cells, while a few inflammatory cells, oligodendroglia, and astrocytic cells revealed occasional TUNEL labeling (Figures 3(e), 3(g), and 3(i)). Sham-infected animals (adult or neonatal) did not show apoptotic labeling.

In canine brains, no neuronal labeling was seen in any of the anatomical areas studied. But several microglial cells in the perivascular zone, endothelial cells lining the blood vessels, and the glial cells in the white matter had labelling by the TUNEL technique (Figure 4(b)). The labeling of inflammatory cells was significantly high in all the canine brains examined compared to mice or human brains (Figure 4(c)). In canine brains, CD68-positive macrophages, surrounding the partially digested neurons and microglial nodules in the vicinity, showed TUNEL labeling suggesting phagocytosis of apoptotic bodies by the macrophage system (Figure 1(d)). The neurons bearing rabies viral antigen failed to show TUNEL positivity in serial sections. In the human brain (6/6 brains), the hippocampus and medulla oblongata revealed occasional TUNEL-labeled inflammatory cells in contrast to canine brain (2/9 cases) (Figure 4(e)).

4. Discussion

The formation and maintenance of body form is dependent on apoptosis, a programmed cell death, which is genetically controlled and plays a vital role in both embryonic development and tissue homeostasis in adults [19, 20]. Apoptosis plays a protective role in eliminating virus-infected cells, which might prove harmful if they were to survive. The process of apoptosis has been observed in a multitude of viral infections [21–26], and the number of correlations between viral pathogenesis and apoptosis continue to grow [8, 27]. Some viruses utilize apoptosis as a mechanism to induce cell death, whereas other viruses have clearly exploited the highly regulated apoptotic cascades by blocking it within the cells they reside in to promote their survival in the host environment. RNA viruses multiply rapidly to produce many virions before the host mounts an effective immune response [28, 29].

In the recent years, neurotropic viruses in particular have been shown to induce apoptosis within the CNS,

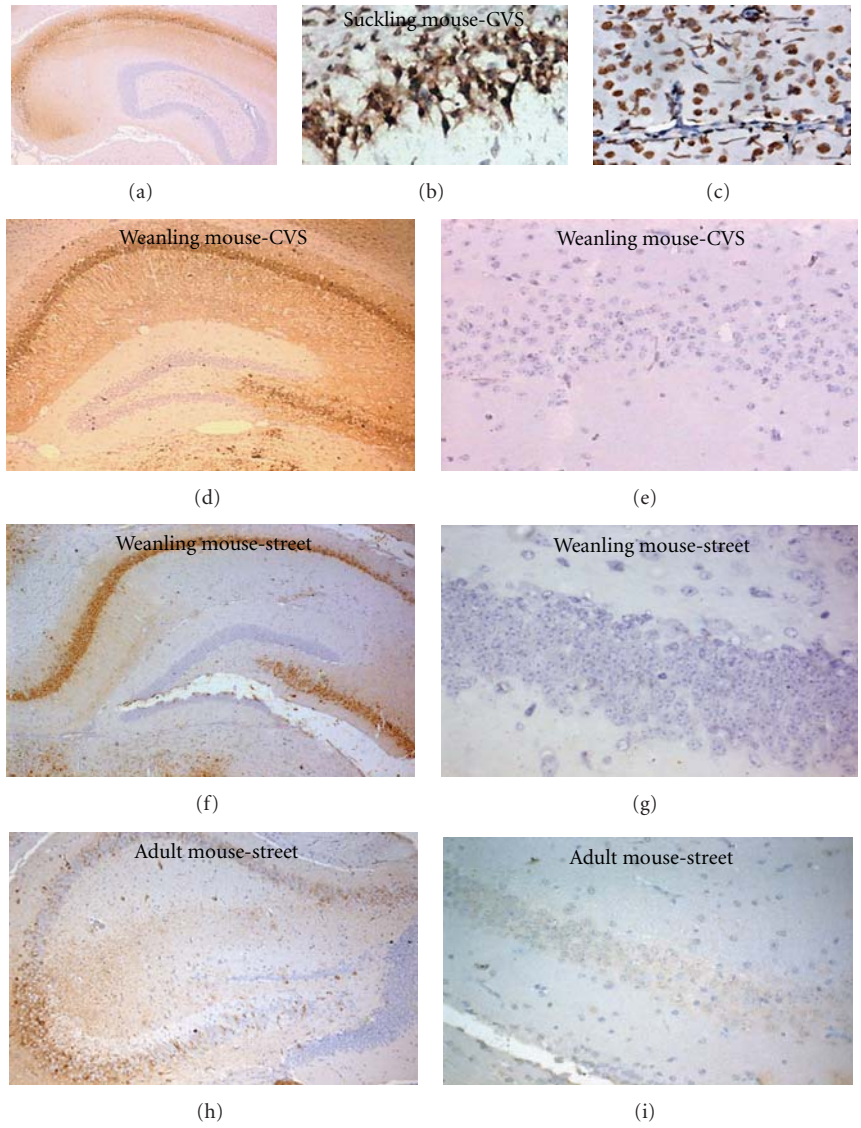

Figure 3: Suckling mouse brain infected with CVS strain of rabies virus shows rabies viral antigen within the pyramidal neurons of hippocampus (a). Infected pyramidal neurons (b) and glial cells (c) reveal apoptosis by TUNEL immunolabeling. Endothelial cells lining vessels are negative. Weanling mouse brain infected intramuscularly with CVS strain of rabies virus (d-e) shows rabies viral antigen pyramidal neurons of hippocampus (d) and absence of TUNEL labeling in these neurons (e). Weanling mouse brain infected intramuscularly with street strain of rabies-virus-labeled neurons in hippocampus (f) and TUNEL labeling is negative (g). Brain from adult mouse infected intramuscularly with street and CVS strain of rabies virus, respectively, labeled the neurons (f, h) but no apoptosis seen by TUNEL technique (g, i).

and depletion of these nonregenerating neuronal cells by apoptosis may result in neurological morbidity [26, 30–32]. This association between viral infection of the CNS and apoptosis has spawned a new area of rabies virus research (Table 3). Researchers initially probed the role of rabies-virus-induced apoptosis in neuronal and nonneuronal cell lines using attenuated laboratory strains of rabies virus and concluded that a variety of commonly used laboratory strains of rabies virus can indeed induce apoptotic cascades within the cell. Apoptosis was initially documented in rat prostatic adenocarcinoma (AT3) cells infected with a highly neurotropic challenge virus standard (CVS) of rabies virus [12]. Thouloze et al. showed that the ERA (Evelyn Rotnycki Abelseth) strain of rabies virus (an attenuated strain with restricted cell tropism and nonneuronal cell infection) was able to induce apoptosis in Jurkat T-cells *in vitro* [33]. The fixed rabies virus strain CVS-11 was found to induce apoptosis of infected mouse neuroblastoma cells, as demonstrated by both DNA laddering as well as TUNEL-positive staining of infected cells [34].

Following this, Jackson and coworkers reported induction of widespread neuronal apoptosis and strong TUNEL labeling in laboratory animals (adult and suckling ICR mice) infected intracerebraly with the CVS strain [12, 35]. Theerasurakarn and Ubol substantiated the presence of apoptosis in CVS-infected suckling mice brain and suggested

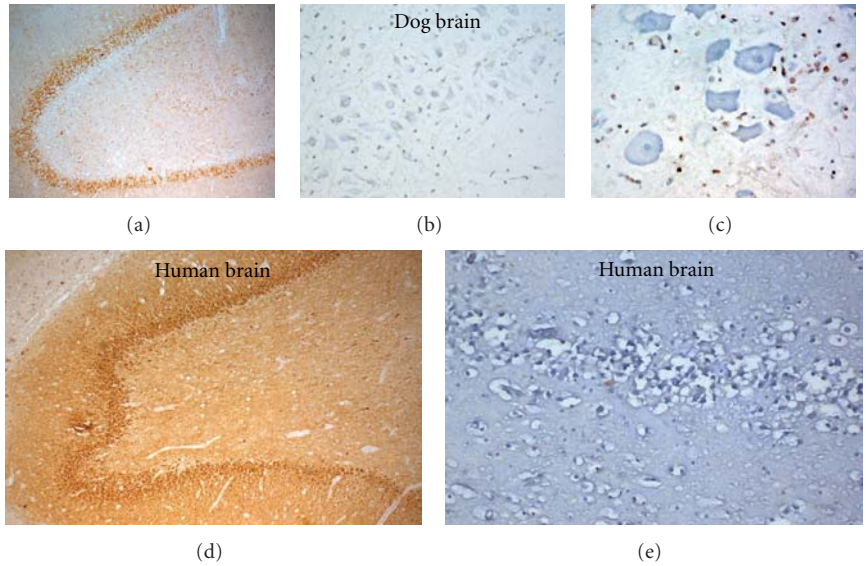

Figure 4: Intense immunolabeling of hippocampal pyramidal cells in canine brain naturally infected with street rabies virus (a) are negative for apoptosis by TUNEL labeling (b). Few inflammatory cells in nuclear area of medulla oblongata are labeled by TUNEL technique (c). No neuronal apoptosis noted in these anatomical areas. Pyramidal neurons in human hippocampus from a case of paralytic rabies infected by street virus showed viral antigen (d) in the absence of TUNEL labeling (e). (a: Immunoperoxidase x40; b: TUNEL ×160; c: TUNEL ×400; D: Immunoperoxidase ×40; E: TUNEL ×160.)

that it was an early event that correlated with disease severity [36]. Examination of brain tissues from Balb/c mice infected with attenuated Pasteur strain of virus (PV), also revealed neuronal apoptosis in both clinically asymptomatic and paralyzed animals.

Mechanism of apoptotic induction has been addressed in few studies. *Bax*-related, caspase-dependent induction of apoptosis was shown rabies infected neuroblastoma cells [34], along with reactivation of Nedd-2, a developmentally downregulated apoptotic gene and other proapoptotic genes [37, 38]. But *in vivo*, although apoptotic cell death has found to be less prominent in *bax*-deficient mice inoculated with CVS or RV194-2 strains, Jackson detected apoptosis in infected brain stem neurons suggesting that other factors may also play a role [39]. Role of adaptive immunity was investigated but ruled out as apoptosis was demonstrable even in immunodeficient mice as well as in humans [15, 40]. The extent of neuronal apoptosis in p75 neurotropin receptor mice when compared and wild-type mice inoculated with CVS strain did not show appreciable differences suggesting the lack of direct role in mediating apoptosis [41].

The observations in the present study is in concordance with other studies in suckling mice infected with CVS strain, but neuronal apoptosis was not found in weanling and adult mice. Similarly Jackson and Park had earlier observed that the numbers of apoptotic neurons in the infected suckling mice hippocampus were significantly higher than in the adult mice [35]. The apoptotic cell death in CVS-infected suckling mice but not adults suggest that the developing, immature brain may be more susceptible to apoptotic cell death.

In the present study, the suckling mice inoculated with the CVS strain of rabies virus (IC route) were studied at different time points of the disease process, and apoptosis was detected only in the terminal stage of the illness correlating with severity of infection with progression of the disease. The weanling and adult mice infected with CVS strain of rabies virus (IC and IM) showed no DNA laddering compared to suckling mouse indicating that apoptotic cell loss is not a significant pathogenetic mechanism in causing morbidity in older animals.

The variable results in published literature could be related to differences in virus strain, route of inoculation, or the strain of laboratory animals used and their differential permissiveness. For instance, Scott et al. demonstrated lack of apoptosis in cerebral cortex and hippocampus of six-week-old mice when inoculated with CVS strain peripherally in mediating apoptosis [42]. Earlier studies had documented widespread neuronal apoptosis in mice inoculated by intracerebral route particularly in sucking mice (Table 3) [35, 36, 39, 40]. Ubol and Kasisith demonstrated that infection of Swiss albino mice with either a bat strain of rabies virus or a primary isolate from a rabid dog resulted in massive apoptotic cell death in the rodent brain as visualized by TUNEL staining [37], while Yan et al. observed very little TUNEL-positive staining in mice infected with bat rabies virus (SHBRV) despite the clinical signs of disease [14]. Reid and Jackson also failed to observe apoptotic neuronal pathology but recorded TUNEL positive reactivity in inflammatory cells following injection of two variants of CVS 24 strain of rabies, (CVS N2C, and CVS B2C) into bats (which induced significant apoptosis in suckling mice and primary neural cultures) [13]. Thoulouze et al. in *in vitro* studies demonstrated an inverse correlation between apoptosis and the neurotropic capacity of a virus strain

Table 3: Review of the published literature.

Sl No	Author year	Cells or tissue and the viral strain used	Viral strain	Conclusion Apoptosis present or not, if so which cells
(1)	Adle-Biassette et al., 1996 [15]	Human tissue	Street virus	Apoptotic neurons identified in the brain stem and hippocampus in the vicinity of inflammatory foci but not in noninflammatory areas
(2)	Jackson and Rossiter, 1997 [12]	Cultured rat prostatic adenocarcinoma (AT3) cells Adult ICR mice inoculated intracerebrally with CVS	Challenge virus standard (CVS) strain of fixed rabies virus	Cultured rat prostatic adenocarcinoma (AT3) cells showed apoptosis (DNA laddering and Bax protein expression). Adult ICR mice showed apoptosis in neurons of hippocampus and cerebral cortex. Apoptosis plays an important role in the pathogenesis of rabies virus infection
(3)	Thoulouze et al., 1997 [33]	Activated murine lymphocytes and the human lymphoblastoid Jurkat T-cell lines	Challenge virus standard (CVS) and attenuated strain ERA	(i) Rabies virus infects lymphocytes, (ii) lymphocyte infection with the attenuated ERA rabies virus strain causes apoptosis but not with CVS, and (iii) apoptosis does not hinder rabies virus production. Apoptosis of infected Jurkat T cells was concomitant with viral glycoprotein expression, suggesting that this protein has a role in the induction of apoptosis
(4)	Ubol et al., 1998 [34]	Neuroblastoma cell line	CVS 11	Apoptosis present (DNA laddering, TUNEL, caspase 1, *Bax*)
(5)	Jackson and Park, 1998 [35]	Suckling mice (6-day-old ICR mice)	Challenge virus standard (CVS) strain of fixed rabies virus, intracerebral inoculation	Widespread neuronal apoptosis (TUNEL staining, increased *Bax* expression) in hippocampus and cerebral cortex, in rabies-virus-infected neurons. Apoptosis was more in suckling mice than in adult mice, explaining the greater neurovirulence of rabies virus in younger mice
(6)	Theerasurakarn and Ubol, 1998 [36]	Suckling mouse brain	CVS 11	Apoptosis detected in neurons (TUNEL, DNA fragmentation) and is the earliest death mechanism detected in these mice
(7)	Jackson, 1999 [39]	Five- to 7-day-old *bax*-deficient mice and their wild-type littermates	CVS or the RV194-2 variant of rabies virus inoculated intracerebrally	Apoptosis was less severe in the cerebral cortex, hippocampus, and cerebellum of the *bax*-deficient mice compared to wild-type mice
(8)	Jackson and Park, 1999 [41]	6-day-old p75 neurotrophin receptor-deficient mice and wild-type mice	CVS (Challenge virus standard strain) of fixed rabies virus inoculated intracerebrally	Widespread apoptosis in brain (TUNEL stain)
(9)	Ubol and Kasisith, 2000 [37]	Adult and suckling mice	Bat strain and a primary canine rabies virus	Expression of Nedd-2 correlated with the appearance of apoptotic nuclei within the brain. Apoptosis required for elimination of virally infected cells
(10)	Reid and Jackson, 2001 [13]	Fruit eating adult bats (*Artibeus jamaicensis*)	CVS-N2c and CVS-B2c (stable variants of CVS-24), inoculated into the right masseter muscle	Apoptosis (DNA fragmentation) not observed in rabies infected neurons with either virus strain

Table 3: Continued.

Sl No	Author year	Cells or tissue and the viral strain used	Viral strain	Conclusion Apoptosis present or not, if so which cells
(11)	Yan et al., 2001 [14]	Mice	Street rabies virus (silver-haired bat rabies virus, SHBRV) and mouse-adapted laboratory rabies virus strain (CVS-24)	Apoptosis (TUNEL staining) was observed in the brain with CVS-24-infected mice but not SHBRV infected mice. Apoptosis is not an essential pathogenic mechanism for the outcome of street rabies virus infection
(12)	Rutherford and Jackson, 2004 [40]	Immunodeficient adult C57BL/6J mice with nude mice (T-cell deficient) and Rag1 mice (T- and B- cell deficient)	Challenge virus standard-11 strain (CVS), intracerebral inoculation	Neuronal apoptosis prominent in immunodeficient mice
(13)	Juntrakul et al., 2005 [49]	Brain and spinal cord of 10 rabies patients	Street virus	Apoptosis present and correlated with the presence of rabies virus antigen
(14)	Sarmento et al., 2005 [45]	ICR Mice (4–6 weeks)	Recombinant RVs with replacement of G gene, and wild-type virus (SHRBV), intracerebral route and intramuscular route	With attenuated RV (IC or IM route), mice showed prominent inflammation and apoptosis and inversely correlated with G protein. With wild-type virus inoculated by IC or IM route, apoptosis was minimal. Glycoprotein-mediated induction of apoptosis limits the spread of attenuated rabies viruses in the central nervous system of mice
(15)	Ubol et al., 2005 [38]	Neonatal mice	Street virus, intracerebral inoculation	Proapoptotic genes upregulated
(16)	Jackson et al., 2006 [48]	Two-day-old ICR mice inoculated in a hindlimb thigh muscle	Recombinant rabies virus vaccine strain SAD-L16 (L16) or SAD-D29 (D29), which contains an attenuating substitution of Arg333 in the rabies virus glycoprotein	Less virulent virus was a stronger inducer of neuronal apoptosis
(17)	Weli et al., 2006 [11]	Cultures derived from the cerebral cortices and hippocampi of 17-day-old mouse fetuses	CVS strain of rabies virus	Apoptotic features and activated caspase 3 expression in cultures. Caspase inhibitors were neuroprotective
(18)	Scott et al., 2008 [42]	Six-week-old mice	CVS strain of fixed virus inoculated in the hindlimb footpad	Few apoptotic cells in the cerebral cortex and hippocampus. (TUNEL labeling and caspase-3 immunostaining)
(19)	Jackson et al., 2008 [16]	12 cases of human rabies (four different countries)	Street virus	No evidence of neuronal apoptosis (TUNEL staining) in cerebral cortex, hippocampus, and brainstem. Caspase-3 immunostaining was absent in neurons, but observed in microglial processes
(20)	Suja et al., 2009 [17]	10 canine brains	Street virus	No neuronal apoptosis (DNA laddering)

in human lymphoblastoid Jurkat T cell lines and proposed that blockage of apoptosis could be a strategy evolved by neurotropic virus to favor its neuroinvasiveness [43]. Baloul and Lafon in an *in vivo* study compared the apoptotic properties of neurotropic CVS strain with nonneurotropic vaccine strains like PV and suggested that T cell mediated apoptosis of neuronal cells in the PV-infected brain prevented virus propagation in the brain [44]. Lack of apoptosis in mice inoculated with wild-type virus (SHBRV) in comparison with attenuated strains was highlighted by Sarmento and coworkers [45] Other viral factors such as glycoprotein expression have been shown to influence pathogenicity of different rabies virus variants and correlate inversely with apoptosis [45–47]. Jackson et al. using recombinant vaccine strain SAD-L16 (L16) or SAD-D29 (D29), which has an attenuating substitution of Arg333 in the viral glycoprotein, demonstrated that the less neurovirulent strains of rabies virus are stronger inducers of neuronal apoptosis [48].

Apoptosis was not detectable in our study when street virus strain was inoculated (IC or IM) into both suckling and adult mice in our study. Due to the long incubation period with the street virus, the suckling mice were almost weanling, by the time they manifested symptoms (12–15 days) of the disease, underscoring the age dependence of the apoptotic phenomenon to cells in the immature brain. Yan et al. also demonstrated the conclusively presence of apoptosis in brains of CVS-24-infected mice but not street virus- (silver-haired bat rabies virus-) infected mice [14]. The number of studies using street virus strain of rabies is limited [14–17, 38, 49] (Table 3). Yan et al. was first to demonstrate lack of apoptosis in mice inoculated with silver-haired bat rabies virus [14]. Jackson et al. convincingly demonstrated that there is no role for neuronal apoptosis in mortality in rabies in natural infection in humans by street viral strain [16]. Two studies have, however, shown presence of apoptosis in humans [15, 49]. The immunocompromised state (HIV-1 positive) of the host, in the case described by Adle Biassette et al. [15], could have induced apoptosis as previously demonstrated in immunodeficient experimental mice [40]. Juntrakul and coworkers in ten cases of human rabies demonstrated apoptosis in spinal cord [49]. Other workers have not replicated this, and the authors themselves suggest that the occurrence of apoptosis diffusely throughout the neuraxis could have been contributed by other factors such as hypoxia and ischemia occurring preterminally. Conspicuous lack of neuronal apoptosis in our previous study on canine brains [17] and the present study concurs with that of Jackson and coworkers [16].

The occurrence of apoptosis resulting from infection with a "street rabies" virus strain was restricted to inflammatory cells but not virus-infected neurons; thus it may not be responsible for mortality of the host [16].

In the natural infection by the rabies virus as observed in the present study, in human, canine, and mice inoculated with street rabies via intramuscular route, neuronal apoptosis was minimal and inconsequential despite the widespread presence of rabies virus in almost all neuroanatomical areas of the brain examined. The street virus has, therefore, evolved very efficient measures to prevent neuronal death by blocking apoptosis, thereby promoting widespread and efficient neuroinvasion by the virus.

5. Conclusion

Prevention of neuronal apoptosis appears to be a subversive strategy developed by the street strain of rabies virus to evade elimination and promote persistent nonlethal infectious cycle, ensuring the long-term survival of the rabies virus in the host. This could account for longer incubation period noted in higher animals. In addition, our observations suggest that street virus triggers apoptosis in inflammatory cells, thereby interfering with release of cytotoxic cytokines and preventing cell lysis. Further studies on the molecular mechanisms, utilized by the rabies virus to prevent apoptosis, will shed light on the pathobiology of rabies viral infection and thereby explain the cause of fatality in these cases.

Acknowledgments

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Review Article

A Morphological Approach to the Diagnosis of Protozoal Infections of the Central Nervous System

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Protozoal infections, though endemic to certain regions, can be seen all around the world, because of the increase in travel and migration. In addition, immunosuppression associated with various conditions, particularly with HIV infection, favors the occurrence of more severe manifestations and failure to respond to treatments. The CNS may be the only affected system; when not, it is often the most severely affected. Despite information obtained from clinical, laboratory, and imaging procedures that help to narrow the differential diagnosis of intracranial infections, there are cases that need confirmation with biopsy or autopsy. Predominant presentations are meningoencephalitis (trypanosomiasis), encephalopathy (cerebral malaria), or as single or multiple pseudotumoral enhancing lesions (toxoplasmosis, reactivated Chagas' disease). The immune reconstitution disease, resulting from enhancement of pathogen-specific immune responses after HAART, has altered the typical presentation of toxoplasmosis and microsporidiosis. In this paper, a morphological approach for the diagnosis of protozoal infections affecting the CNS (amoebiasis, cerebral malaria, toxoplasmosis, trypanosomiasis, and microsporidiosis) is presented.

1. Introduction

Protozoa are single-cell organisms widely distributed in nature. Protozoal infections, though endemic to certain regions for reasons of climate and availability of intermediate hosts to transmit them to man, are also seen outside their original geographical areas, probably facilitated by the increase in international travel and migration of people from their native countries [1–3].

These infectious diseases should be largely known, particularly because immunosuppression associated with HIV infection, solid organ, or bone marrow transplant with long-term immunosuppression caused by medications, favor the occurrence of more severe clinical manifestations and failure to respond to specific treatments. A significant proportion of these cases have been described due to immigrants coming from tropical countries to nontropical countries, and increased awareness of these diseases is needed among health professionals dealing with such patients [1, 4, 5].

For a number of these organisms, the nervous system is only one of the many systems involved; however, this localization may often be the most severe and incompatible with the survival of the patient [2, 6].

Although much information is obtained from clinical examination, laboratory, and imaging procedures [7, 8], all playing a crucial role in identifying and narrowing the differential diagnosis of intracranial infections, there are still cases that need biopsy or autopsy studies to confirm the diagnosis [2, 3, 9].

Examination of blood and cerebrospinal fluid (CSF), through direct observation, culture, serology, and molecular diagnosis (including the use of the polymerase chain reaction (PCR)), is extremely important, but tissue samples provide morphological evidence of infection, as well as the substrate for culture and molecular diagnostics. Genetic polymorphisms are increasingly recognized as factors accounting for variation in disease frequency and presentation between individuals [2]. However, a significant number of the diseases are only—and often inevitably so—diagnosed after death.

The autopsy brain examination is critical in retrospective diagnosis and audit, so that similar presentations in the future may be more accurately managed. It is important to take into account that disease patterns have changed over time, so that what is presented to neuropathologists for evaluation has shifted dramatically in the last two decades [3, 10].

The introduction of highly active antiretroviral therapy (HAART) has improved both the clinical and radiologic findings in HIV-infected patients and reduced the number of opportunistic infections. However, the immune reconstitution inflammatory syndrome (IRIS), or immune reconstitution disease (IRD), the clinical presentation or deterioration of infections resulting from enhancement of pathogen-specific immune responses after the institution of HAART, has altered the typical presentation of some protozoal infections involving the central nervous system (CNS), such as toxoplasmosis and microsporidiosis [4, 11].

The predominant presentation of protozoal infections in the CNS may be as meningoencephalitis (e.g., African trypanosomiasis), encephalopathy, such as in cerebral malaria, or as single or multiple space-occupying enhancing lesions in a pseudotumoral form, such as in toxoplasmosis [12, 13].

In this paper, a morphological approach for the diagnosis of most protozoal infections that affect the CNS is presented with a brief description of the etiology, epidemiology, and relationship with immunodeficiency.

A brief taxonomic classification of the major protozoan infections of the CNS is presented in Table 1.

Table 1: Protozoal infections that may affect the central nervous system.

Amoebiasis
Cerebral abscess
Entamoeba histolytica
Primary amoebic encephalitis (free living *amoebae*)
(a) Primary amoebic meningoencephalitis
Naegleria fowleri
(b) Granulomatous amoebic encephalitis
Acanthamoeba spp.
Balamuthia mandrillaris
Leptomixed amoebas
Sappinia diploidea
Cerebral malaria
Plasmodium falciparum
Toxoplasmosis
Toxoplasma gondii
Trypanosomiasis
African trypanosomiasis (Sleeping sickness)
Trypanosoma brucei gambiense
Trypanosoma brucei rhodesiense
South American trypanosomiasis (Chagas disease)
Trypanosoma cruzi.
Microsporidiosis
Encephalitozoon spp
Leishmaniasis
Leishmania spp

Note that cerebral leishmaniasis is exceedingly rare, and microsporidial infections have become apparent as significant human CNS diseases only in the last decades.

2. Amoebiasis

2.1. Cerebral Amoebic Abscess. Cerebral amoebic abscess caused by *Entamoeba histolytica* infection, is a rare global disease, not related to immunodeficiency that causes proctocolitis with bloody dysentery, and liver abscesses and rarely cerebral abscess through haematogenous spread from liver. Transmission is by ingestion of cysts in infected faeces [14, 15].

2.1.1. Macroscopic Appearances. The cerebral lesion is usually single and located in the cortical gray matter, basal ganglia, or at the junction between cortex and white matter. Early lesions appear as small foci of hemorrhagic softening that become necrotic, with yellow-green centers, and later cavitate. The walls are irregular and there is no evidence of encapsulation. Occasionally there are multiple abscesses [2, 16].

2.1.2. Microscopic Appearances. Cerebral amebic abscesses have an inner zone of necrotic tissue and a broad outer zone with prominent congestion and vascular proliferation. A reactive gliosis and an infiltrate of lymphocytes, plasma cells, macrophages, and some neutrophils are seen in the surrounding brain. Trophozoites can usually be identified in the abscess wall, around vessels, in the necrosis, and at the advancing edge of the lesion. In certain occasions, it may be difficult to distinguish *E. histolytica* trophozoites within this tissue from macrophages. The trophozoites are spherical or oval, 10–60 μm in diameter, with granular eosinophilic, sometimes vacuolated cytoplasm, a round single nucleus with a small central karyosome and peripheral chromatin. Many have phagocytosed erythrocytes and occasionally pseudopodia can be seen. The amoebae are PAS positive, since the cytoplasm contains glycogen (Figure 1). Specific antisera can also be used to identify them by immunocytochemistry [2, 16].

2.2. Primary Amoebic Encephalitis (Free Living Amoebae). Infection due to free-living amoebas has increased significantly during the last decades especially in developing countries. Although having in common the ability to run a life independent from the host and the fact that cerebral involvement may be fatal, free living amoebas differ with regard to their epidemiology [17].

Acute CNS infection due to Naegleria fowleri, which ends in death within 2–7 days, is termed primary amoebic meningoencephalitis and is not related to immunodeficiency.

Subacute or chronic CNS infections due to *Acanthamoeba spp, Balamuthia mandrillaris, Leptomixed amoebas, and Sappinia diploidea* are termed granulomatous amebic encephalitis [18, 19], a condition associated with immunodeficiency and also with other chronic debilitating conditions, such as malnutrition and diabetes [20, 21].

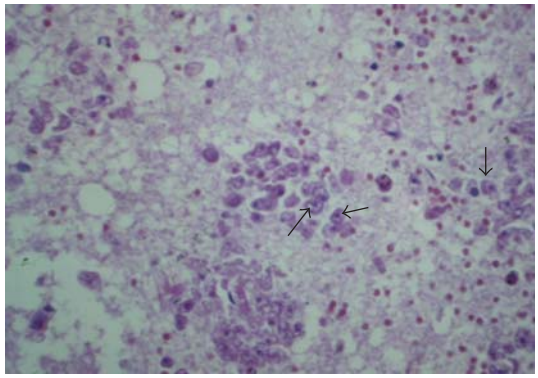


Figure 1: *Entamoeba histolytica* trophozoites are spherical or oval, with granular, sometimes vacuolated cytoplasm and a round single nucleus. The amoebae are PAS positive (arrows).

2.2.1. Primary Amoebic Meningoencephalitis. Naegleria fowleri is a free-living amoeba encountered in soil, fresh water, and hot springs. It is found in swimming pools, lakes, and rivers, which are commonly used during the summer months, when the parasite preferentially multiplies. It has a worldwide distribution, occurring as individual cases or small outbreaks. Although the majority of case reports are from the USA, Australia, and Central America this may reflect recognition of the disease. The parasite either exists as a freely motile trophozoite, or in unfavourable circumstances can encyst, hence its ability to survive outside the host. Both the flagellar trophozoite and the cyst forms are infective. The affected individuals are usually young and healthy. The organism invades the nasal mucosa and enters the brain by travelling along the olfactory nerves [17, 21].

(1) Macroscopic Appearances. The appearances are those of a fulminant acute meningoencephalitis with a characteristic haemorrhagic necrosis of the olfactory bulbs, tracts, and the adjacent parts of the frontal and temporal lobes. The brain is swollen with a hemorrhagic exudate all over the meninges [21].

(2) Microscopic Appearances. Scant inflammation consisting of polymorphonuclear and variable mononuclear infiltrate with focal hemorrhage is seen in the meninges, extending along the Virchow-Robin spaces, associated with haemorrhagic necrosis of the grey and white matter. *N. fowleri* trophozoites measuring 8–15 μm are present in the subarachnoid space and around vessels in the necrotic parenchyma The cytoplasm is vacuolated resembling macrophages, but can be distinguished from them by their vesicular nucleus with its prominent central nucleolus [2, 16] (Figure 2).

2.2.2. Granulomatous Amoebic Encephalitis (GAE). Acanthamoeba castellanii, A. polyphaga, and *Balamuthia mandrillaris,* ubiquitous within the environment in both soil and water, are the most frequent free-living amoebae that cause this pattern of disease. Infection can occur at any time during the year, and mortality due to neurological complications is high. The main risk factors are HIV infection, lymphoma, malnutrition, cirrhosis, and diabetes [2, 21, 22]. Amoebae enter into the lungs via the nasal route, followed by haematogenous spread from where they cross the blood-brain barrier and enter into the CNS. Skin lesions may provide direct entry into the bloodstream, bypassing the lower respiratory tract. The olfactory neuroepithelium may provide an alternative route of entry into the CNS [23].

(1) Macroscopic Appearances. The brain is usually swollen, covered by a diffuse leptomeningeal exudate and shows foci of softening, particularly in the anterior part of the cerebral hemispheres, the thalamus, brain stem, and cerebellum. Lesions are haemorrhagic and necrotic (Figure 3(a)).

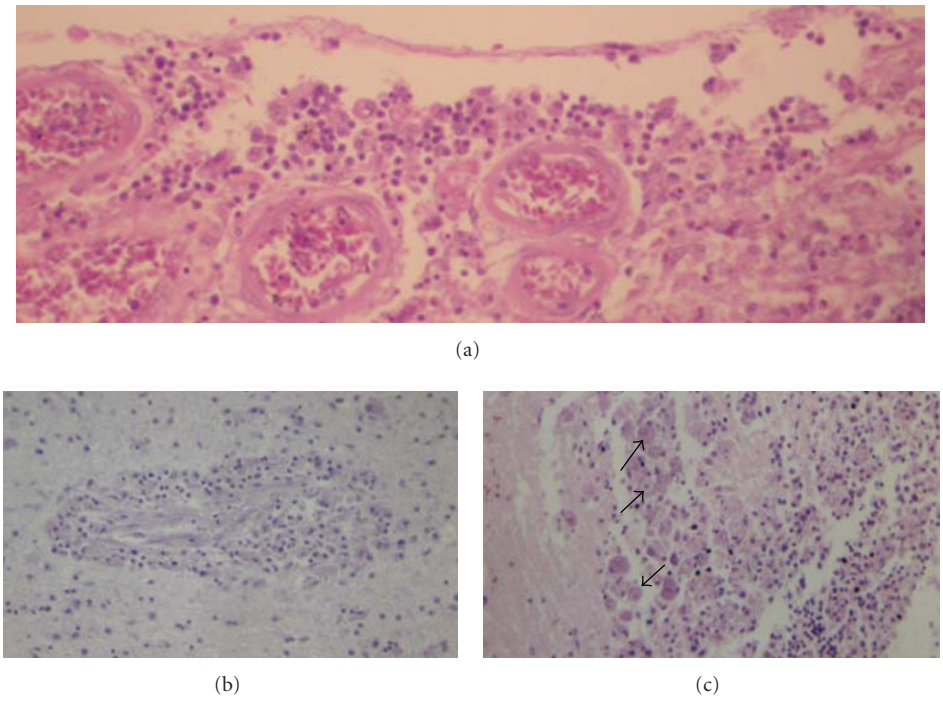
(2) Microscopic Appearances. The meninges and the parenchyma show foci of chronic granulomatous inflammation that tend to be angiocentric and may be necrotizing. The cell reaction is combined acute and chronic, but lymphocytes, macrophages, plasma cells, and foreign body and Langhans-type multinucleated giant cells predominate (Figures 3(b) and 3(c)). *Acanthamoeba* and *Balamuthia* species can encyst. Trophozoites and cysts may be clustered around inflamed vessels that may show fibrinoid necrosis, and also in areas relatively free of inflammation. The trophozoites are 14–40 μm in size that have a pale eosinophilic granular cytoplasm a prominent vesicular nucleus with a dense central nucleolus. Cysts slightly smaller have a double thicker cell wall. Cysts and trophozoites are well demonstrated with the Giemsa and PAS staining (Figure 4). The surrounding brain tissue is necrotic and shows marked astrocytosis and microgliosis. The morphology of the various amoebae that cause GAE is similar. The differentiation can be made by specific immunohistochemistry and molecular diagnostic methods [2, 16, 22].

3. Cerebral Malaria

Malaria is caused by infection with the protozoan parasite *Plasmodium* transmitted by the anopheline mosquito. Four species cause human disease: *Plasmodium falciparum, P. vivax, P. malariae,* and *P. ovale*.

Of these, *P. falciparum* causes the most severe morbidity and mortality and is responsible for the syndrome of cerebral malaria (CM), an important and complex neurological infection that is not related to immunodeficiency. One-third of the world's population is exposed to *P. falciparum* infection, the largest number being in Africa; but South East Asia, India, Central, and South America are also endemic zones [24, 25].

It is associated with widespread morbidity and mortality, especially in infants and children, as well as during pregnancy, producing a high morbidity between both mother and fetus. CM is a clinical rather than a pathological diagnosis and should be considered in the differential diagnosis of any patient who has a febrile illness with impaired consciousness who lives in or has recently traveled to malaria endemic areas.



(a)

(b) (c)

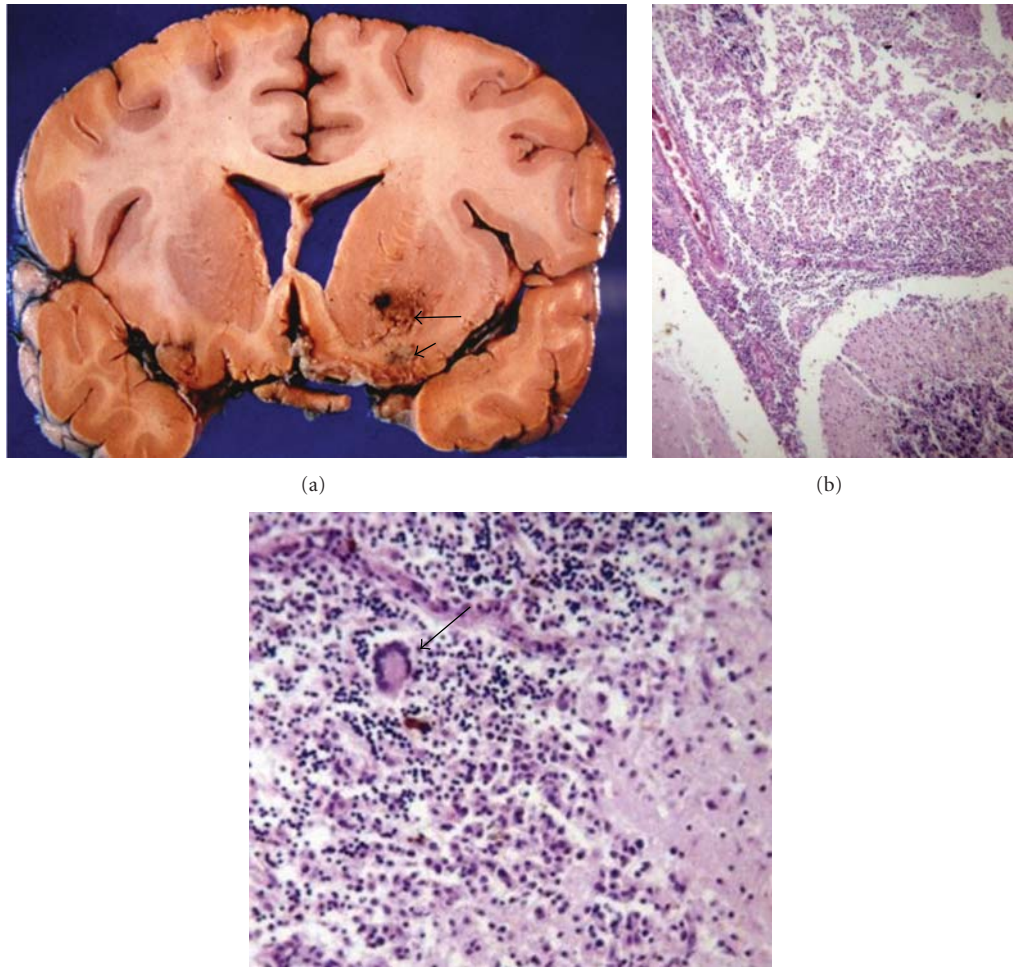
Figure 2: Primary amoebic encephalitis: scant meningeal inflammation (a) extending along the Virchow-Robin spaces (b). *N. fowleri* trophozoites *(arrows)* present within the necrotic tissue resemble macrophages (c). H&E.

Morphologically, the common feature is the heterogeneity in neuropathological findings that is in part due to variations in host immunity, the time of death, degree of treatment, and supervening pathology from severe malaria in other organs. Paediatric cerebral malaria differs from those in adults and there is also geographical variation in the clinical and morphological presentation of different populations in Africa and South-East Asia. Genetic differences among the parasites, the degree of host immune response, and underlying genetic polymorphisms, may account for the differences [26].

3.1. Macroscopic Appearances. The brain weight may be increased by cerebral swelling, which is variable but usually mild and symmetrical in both adults and children. The meninges are congested and the external colour of the unfixed brain is often a characteristic dusky dark red. In severe cases, petechial haemorrhages are seen on the cortical surfaces. On slicing, there may be obliteration of the sulci and flattening of the gyri. In patients with coexistent severe anaemia, the surface can be pale, whereas in a heavily parasitized brain, the deposition of malaria pigment can give a slate gray color due to the presence of abundant malarial pigment. The cerebral cortex may have an abnormal dusky pink color due to marked congestion. The white matter often contains petechial hemorrhages, prominent in the subcortical white matter, corpus callosum, cerebellum, and brain stem (Figure 5); they are also seen in the cerebellar grey matter in children and are frequent in those whose disease evolves over several days and who have been kept alive in medical care; they are less common and frequently absent in those who have died acutely of cerebral malaria, without medical attention [2, 16].

3.2. Microscopic Appearances. The central neuropathological feature of CM is the sequestration of parasitized red blood cells (PRBCs) in the microvasculature, which can most easily be recognized under high dry lens or oil immersion, as small ring forms. They are also recognized by the intraerythrocytic pigment (haemozoin) body in the later trophozoite or schizont stages, since erythrocytes infected with the late maturing stages of the parasite disappear from the free circulation, causing a drop in the observed peripheral parasitemia. The specific binding of PRBC to the lining of blood vessels is confirmed with electron microscopy which shows electron dense knob-like protrusions on the surface of infected erythrocytes and at sites of attachment to vascular endothelium [27]. Parasites adhere to specific receptors in the cerebral microvasculature.

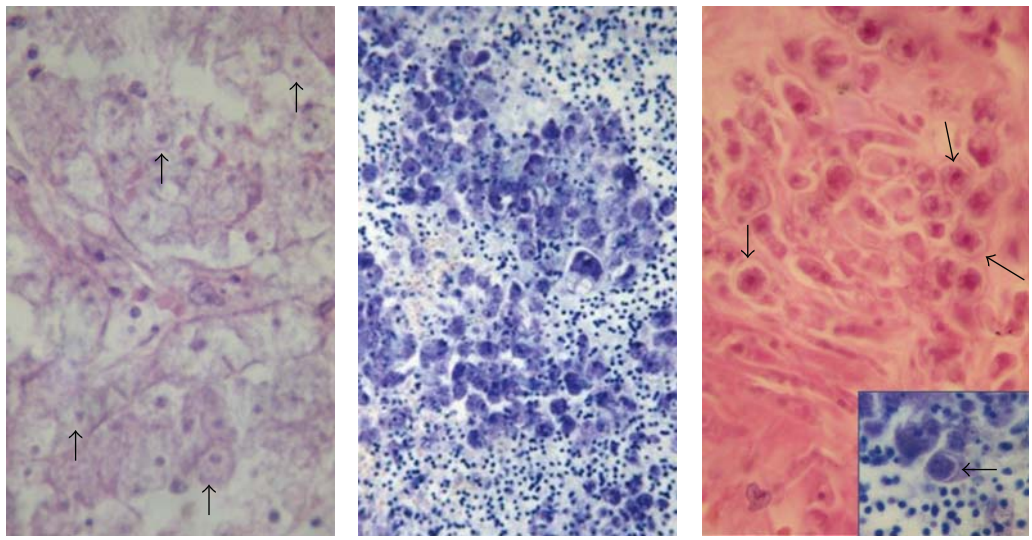
Haemozoin pigment deposition occurs microscopically in the lining of the blood vessels (Figure 6), especially in the meninges and choroid plexus. The pigment may obscure the parasites in the trophozoite stage and can appear similar to formalin pigment, although usually forming smaller and darker granules. If there is any doubt over the presence or absence of PRBC, treatment of the slide with picric acid to remove any pigment will then reveal the parasites more clearly. If the brain is examined after 3-4 days of standard therapy, few sequestered PRBC may be seen. In this case, the presence of residual malarial pigment is a diagnostic clue.



(a) (b)

(c)

Figure 3: Granulomatous amoebic encephalitis. (a) Foci of haemorrhagic softening (arrows). (b) Necrotic cerebellar folium (b) with a giant cell (c) (arrow), H&E.



(a) (b) (c)

Figure 4: *Acanthamoeba* and *Balamuthia* spp have similar morphology. (a) Trophozoites have a pale granular cytoplasm (arrows), that stain well with Giemsa (b) Cysts have a double thicker cell wall (c), also shown in the insert (arrows). (a) and (c), H&E; (b) and insert, Giemsa.

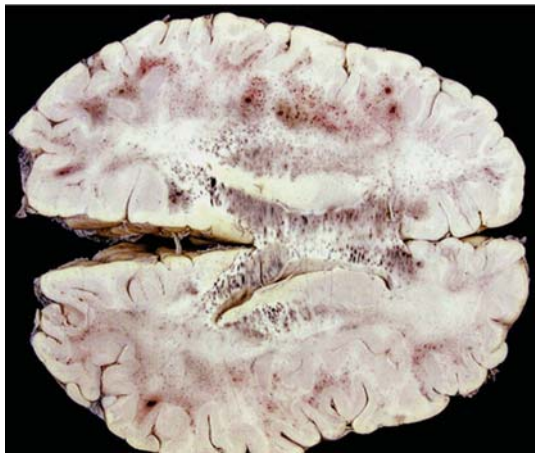


Figure 5: Cerebral malaria: the white matter contains petechial hemorrhages, prominent in the subcortical white matter and *corpus callosum*.

Rupture of infected erythrocytes can lead to an inflammatory process within and around brain capillaries, confirmed with immunocytochemical and molecular biological studies, though cellular inflammation around vessels and in the parenchyma is not a usual feature. If a patient has died after some days of treatment, haemozoin-laden monocytes and neutrophils can be seen in some blood vessels; these cells phagocytose erythrocyte ghosts' adherent to vascular endothelial cells. Patchy endothelial cell activation characterized by swelling, focal endothelial cell damage, and necrosis is sometimes seen, most often associated with incipient areas of haemorrhage. Widespread astroglial activation is seen surrounding blood vessels and microglial nodules may be seen within the brainstem. Markers of microglial cell activation such as CD68, HLA class II antigens, and scavenger receptors are upregulated in the brain in CM. Endothelial cell activation is also evident by the increased expression of intercellular adhesion molecule-1 (ICAM-1). There may be meningeal infiltration by lymphocytes and macrophages [28].

Other neuropathological features include petechial hemorrhage in the brain parenchyma, ring hemorrhages, and Dürck's granulomas. Petechial or larger hemorrhages can occur in any part of the brain, but are most common in the white matter and may surround necrotic arterioles and venules (Figure 7). Ring hemorrhage consists of a series of concentric rings surrounding a central necrotic cerebral vessel. The outermost ring contains a mixture of parasitized erythrocytes, free pigment, and host monocytes, with an inner layer of uninfected erythrocytes and gliosis surrounding the vessel. The other lesion peculiar to the brain in malaria is the Dürck's granuloma, which are multiple circumscribed diffusely scattered cellular reaction (collections of astrocytes and microglia containing iron pigment), probably related to resorption of ring hemorrhages. It seems that ring hemorrhages and Dürck's granulomas may represent a temporal spectrum of the same lesion, granulomas being what remains after the red cells, infected and uninfected, are cleared from hemorrhage and this begins to be organized by host response. Similarly, petechial hemorrhages may be the result of vessel rupture in areas of no sequestration, where parasites and their products cannot, or have not yet elicited any host reaction [2, 16].

Ischemic changes are not great enough to account for coma as a purely hypoxic event. The reversibility of coma in CM and the large numbers of patients who recover complete neurological function would argue against widespread permanent hypoxic damage, or reperfusion injury, as a pathological mediator in most cases. However, focal necrosis of the brain parenchyma occurs in the white matter. This is ischaemic in origin or follows the petechial haemorrhage and elicits the formation of the Dürck's granuloma.

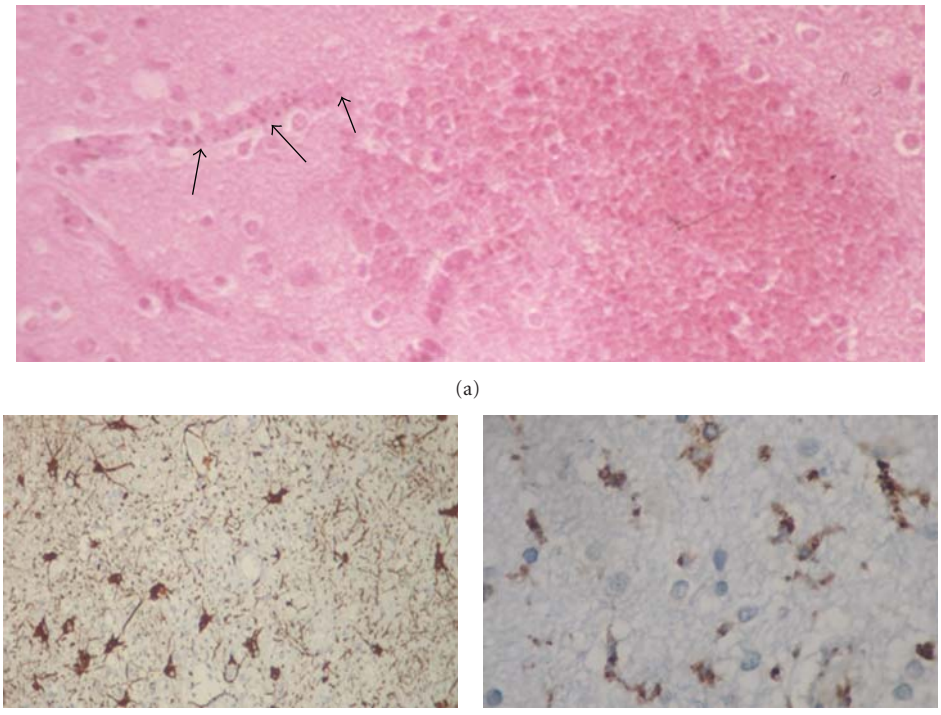
Associated axonal damage can sometimes be demonstrated using β-amyloid precursor protein staining [29, 30].

4. Toxoplasmosis

Toxoplasmosis is caused by infection with coccidian parasite *Toxoplasma gondii*. It is distributed globally, with the cat as the definitive host, but any warm-blooded animal, including man, can be an intermediate host. Infection can be acquired in uterus, with severe damage to the developing brain and eye, but most people become infected after childhood and the primary infection is usually clinically silent and latent. Significant disease, of which CNS pathology is the most common and important, happens if the latent infection reactivates when the immune system is compromised for various reasons. Cerebral toxoplasmosis is one of the most frequent opportunistic infections associated with HIV-related immunodeficiency [31]. Presentation as mass lesion is the most common pattern. This requires differentiation from other AIDS-associated mass lesions occurring in the CNS; mainly AIDS-related lymphoma, although progressive multifocal leukoencephalopathy, cerebral tuberculoma and other CNS tumours should also be considered, since cerebral toxoplasmosis can also occur in combination with these other conditions [32].

4.1. Macroscopic Appearances. The brain contains multifocal single or multiple necrotizing space-occupying lesions of variable sizes. The mass lesions can be located both above and below the tentorium (Figure 8). The basal ganglia and the gray-white matter cortical junctions are often involved, but any part of the brain may be affected. There may be associated hemorrhage. Older lesions are cystic due to resorption of necrotic material (Figure 9). Occasionally brain involvement results in an encephalitic process without obvious focal lesions on macroscopic examination [2, 16].

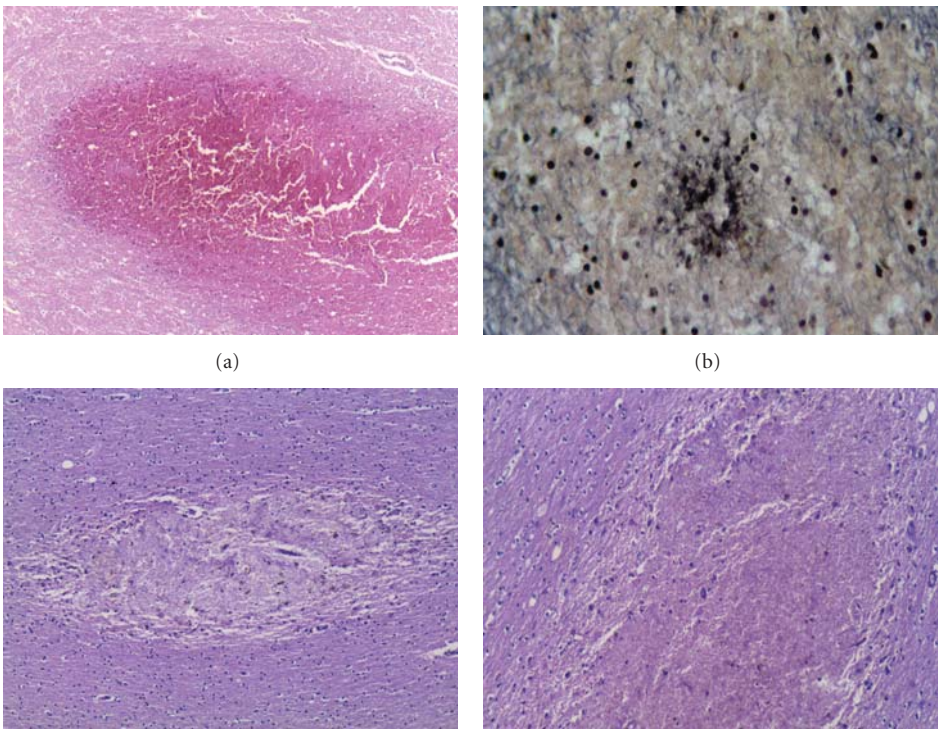
4.2. Microscopic Appearances. There is much heterogeneity of CNS toxoplasma lesions, with overlapping patterns and sometimes temporal heterogeneity. The basic process is cell infection and associated inflammation, forming microglial nodules with surrounding astrocytosis. Necrosis of infected cells and surrounding tissues is usual, leading to expansion of the necrotic foci into the mass lesions that are usually seen.



(a)

(b) (c)

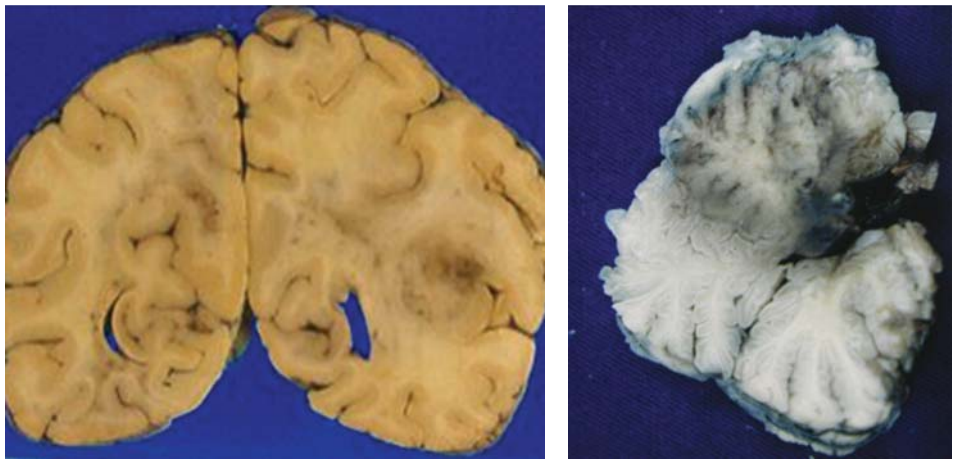
Figure 6: (a) Haemozoin pigment deposition in the lining of the blood vessels (arrows). The pigment may obscure the parasites in the trophozoite stage and can appear similar to formalin pigment, H&E. (b) GFAP and (c) CD68 immunostainings demonstrate astroglial and microglial cell activation, respectively.



(a) (b)

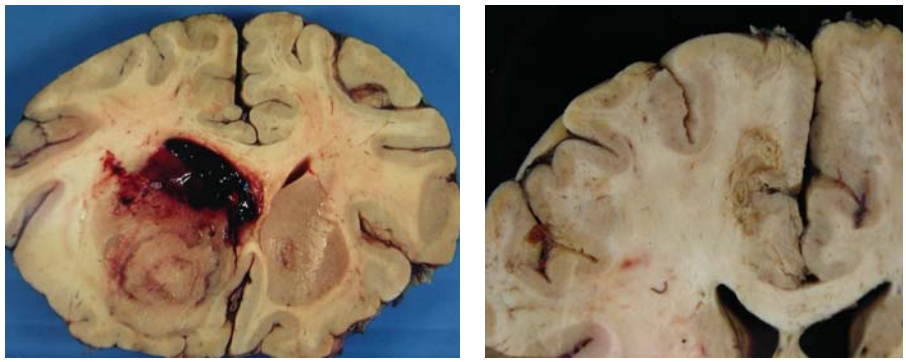
(c) (d)

Figure 7: Histology of acute petechiae (a) that may surround necrotic arterioles and venules (b). After resorption (c, d) there may be diffusely scattered cellular reaction after the red cells, infected and uninfected, are cleared from hemorrhage. (a, c, d) (H&E) (b) (PTAH).



(a) (b)

Figure 8: Toxoplasmosis: mass lesions in the right occipital lobe and cerebellar vermis.



(a) (b)

Figure 9: Toxoplasmosis: (a) acute mass lesion in the basal ganglia with associated haemorrhage. (b) Older lesion in process of organization.

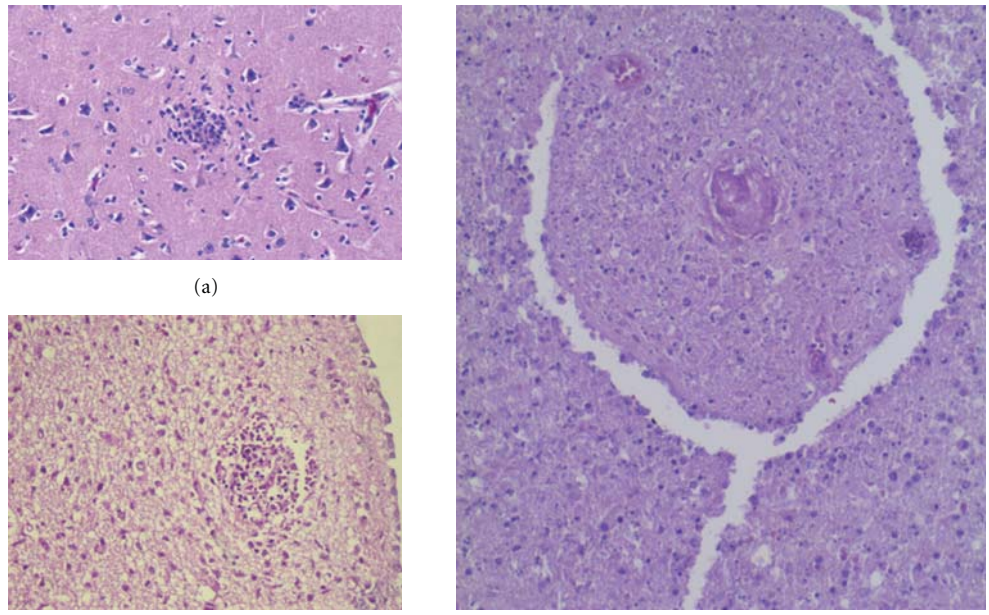
The necrosis is typically coagulative and "dirty", with abundant fragments of nuclear debris (some of which are actually toxoplasma tachyzoites). Around the necrosis there are mononuclear and polymorphonuclear inflammatory cells, newly formed capillaries, edema, reactive astrocytes, and microglia. Vessels are surrounded or infiltrated by lymphocytes and macrophages with the appearances of a vasculitis, occasionally with fibrinoid necrosis, intimal proliferation, and thrombosis, with the features of endarteritis obliterans. Affected vessel may rupture, causing perivascular or larger haemorrhage (Figure 10).

Intracellular and extracellular Toxoplasma tachyzoites (also known as endozoites or trophozoites) and pseudocysts (containing large numbers of bradyzoites, also known as cystozoites), may be easily found with hematoxylin and eosin staining, in the inflamed tissue around the necrosis. Their frequency varies, sometimes abundant, particularly pseudocysts, but on occasions are scanty as in treated lesions, when immunocytochemical staining with anti-*T. gondii* antibodies is useful in identifying parasites. Tachyzoites are oval- or crescent-shaped and measure 2–4 μm. Those within cells may be clustered together (in vacuoles or larger pseudocysts measuring 20–100 μm in diameter) or may appear to lie free in the cell cytoplasm (Figure 11).

In chronic, treated lesions, the central area of coagulative necrosis is well demarcated, may become cystic, containing macrophages (Figure 12), and surrounded by microglial nodules and reactive astrocytes. Organisms are scanty or absent and immunostaining may or may not identify residual antigen in these lesions. Healed or end-stage (burnt out) lesions, when tissue reaction is no longer available, are common presentation nowadays, particularly in countries where the treatment is effective. With the antiretroviral therapy some lesions are associated with the IRIS.

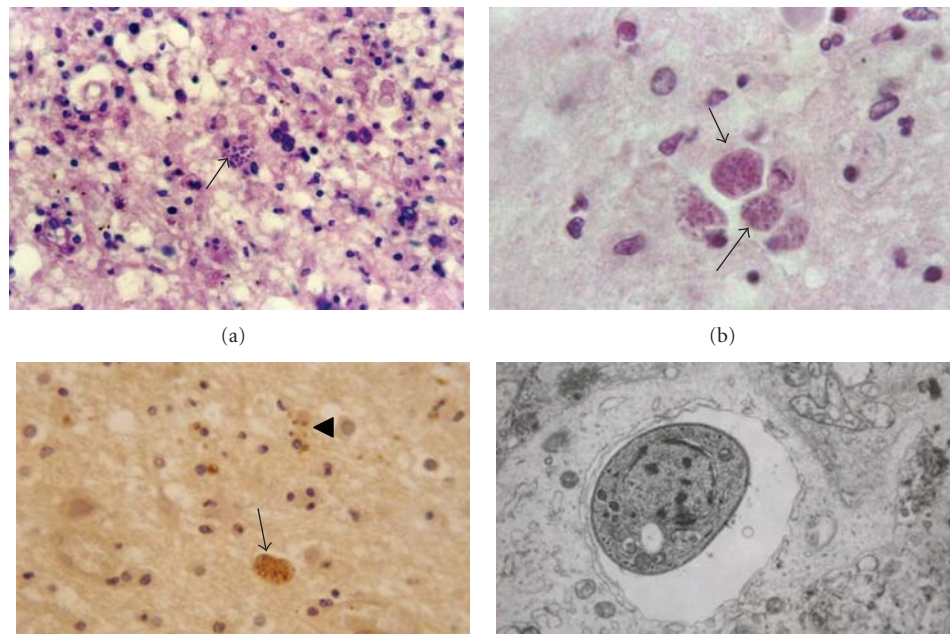
Cerebral toxoplasmosis occasionally causes a diffuse non-necrotizing encephalitic pattern with scattered microglial nodules that include parasites and astrocytosis involving both gray and white matter. In a less frequent peri-ventricular pattern, there is a rim of necrosis up to 1 cm thick along the lateral and third ventricles, where abundant parasites are visible [2, 16].

4.3. Congenital Toxoplasmosis. It is rare (less than 5 cases per 100,000 live births), occurs only if maternal infection is



(a) (b) (c)

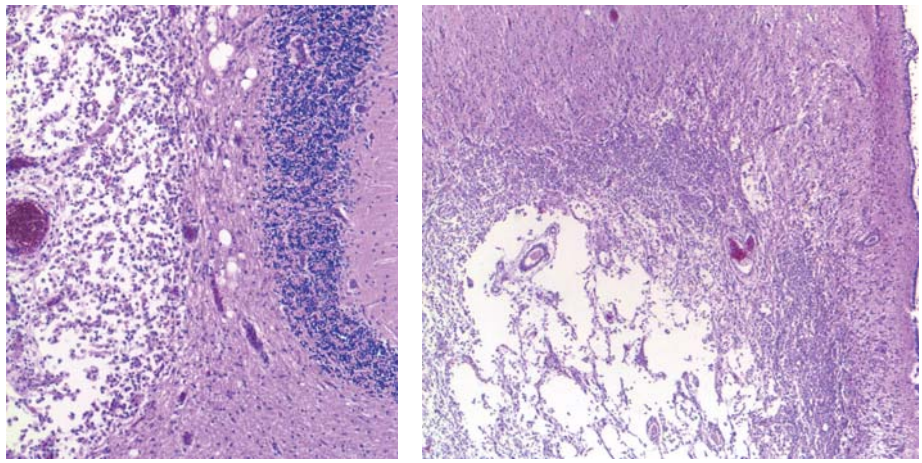
Figure 10: Toxoplasmosis—Microglial nodules (a, b) and "dirty" coagulative necrosis (c) containing a necrotic vessel with the features of endarteritis *obliterans* and thrombosis. H&E.



(a) (b) (c) (d)

Figure 11: (a) Extracellular Toxoplasma tachyzoites (arrow) in the inflamed tissue around the necrosis. (b) Pseudocysts (arrows); (c) immunocytochemical staining with anti-*T. gondii* antibodies identifying tachyzoites (arrowhead) and pseudocysts (arrow). (d) At ultrastructural level a parasite within a vacuole in the cell cytoplasm (a, b), H&E.

acquired during pregnancy, and is transmitted through the placenta [33]. The risk of maternal exposure is geographically variable and may occur at any time during pregnancy, although the highest risk is after the first trimester [34, 35], rising to about 30% and late in the third trimester it approaches 100%. However, a fetal *Toxoplasma* infection early in pregnancy is likely to cause major disruption of CNS organogenesis with resulting fetal death or hydrops and severe cerebral abnormalities. Parasites proliferate and spread widely in the absence of circulating antibody. The severity of



(a) (b)

Figure 12: Toxoplasmosis. Chronic, treated, cystic lesions containing macrophages in the cerebellum (a) and periventricular regions (b).

complications declines with advancing pregnancy, although premature delivery, chorioretinitis, minor brain calcifications, and even fetal death may still result from late infection [33, 36].

4.3.1. Macroscopic Appearances. Macroscopic abnormalities occur in the more severe cases and include multifocal or confluent areas of necrosis, particularly in the periventricular and subpial regions. The brain may be collapsed because of total parenchymal destruction secondary to vascular thrombosis. Periventricular and periaqueductal ulceration, hydrocephalus, or even hydranencephaly may occur. Microcephaly is found in cases with severe brain destruction. Foci of calcification may be scattered throughout the brain in contrast to the predominantly periventricular calcification of congenital CMV [2, 16].

4.3.2. Microscopic Appearances. Necrotic areas are usually associated with lipid-laden macrophages, lymphocytes, and a few neutrophils, which are also present in the leptomeninges. Contact with circulating antibody passively transferred from the mother may account for perivascular inflammation and thrombosis. The adjacent brain tissue may contain microglial nodules. Toxoplasma tachyzoites and cysts can be seen in the meningeal exudate, around the necrotic lesions, and are particularly numerous near the ventricular cavities. They are hard to detect within frankly necrotic areas. Antitoxoplasma antibodies detect both free (tachyzoite) and encysted (bradyzoite) organisms. The foci of necrosis eventually tend to undergo mineralization, although residual toxoplasma cysts can still be found. Ependymal granulations and gliosis may lead to aqueduct stenosis and obstructive hydrocephalus [2, 16].

5. Trypanosomiasis

Trypanosomes are hemoflagellates, that causes disease in large, but geographically restricted, parts of the world. There are three species of *Trypanosoma (T. brucei gambiense and rhodesiense and T. cruzi),* that affect man, all transmitted by blood-feeding insects. Though morphologically similar in their trypomastigote blood form, they give rise to quite different diseases in Africa and South America, respectively [37, 38].

5.1. Human African Trypanosomiasis (Sleeping Sickness). Human African trypanosomiasis (HAT) is caused by two blood parasites *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. Morphologically identical but causing significantly different clinical syndromes, they are found in west Africa (*T. b. gambiense*) and in eastern and southern Africa (*T. b. rhodesiense*) [39]. *T. b. rhodesiense* is a zoonosis, cattle being the reservoir (with significant veterinary consequences).

HAT is transmitted from man to man or cattle to man by *Glossina* spp tsetse flies. It is estimated that millions of people are at risk of infection, including tourists visiting game parks in East and Central Africa. Both infections cause a systemic and meningoencephalitic syndrome in man in late phases of the disease, with high mortality when untreated. Involvement of the central nervous system (CNS) usually follows 3-4 weeks after infection by *T. b. rhodesiense*, whereas it takes many months or years in case of *T. b. gambiense*. The CNS manifestations are protean, nonfocal, and easily misdiagnosed when the patient is encountered out of context, as in migrants to nonendemic countries. The early acute disorder, usually more severe in case of *T. b. rhodesiense* infection, has little or no impact on the CNS, while infection by *T. b. gambiense* is responsible for subacute or chronic meningoencephalitis [40, 41].

5.1.1. Macroscopic Appearances. There are few postmortem reports examining CNS material from human cases available. Macroscopic changes may be scanty or absent. The leptomeninges are congested and may be cloudy, with slightly opaque fluid, more evident at the base and over the cerebellum.

The brain is swollen and congested. No obvious abnormalities are seen on brain section.

Patients who have been treated with melarsoprol may develop acute hemorrhagic leukoencephalopathy, which occurs in 5–10% of late stage treated patients. It is suggested that this reactive lethal encephalopathy is triggered by the viable organisms remaining within the brain after administration of insufficient doses of drugs [16, 42].

5.1.2. Microscopic Appearances. Histological features are those of a diffuse meningoencephalitis consisting of lymphocytes, including large numbers of B cells, plasma cells, and histiocytes surrounding blood vessels and infiltrating the subarachnoid spaces. The vessel walls are inflamed but not necrotic. Lymphocytes and plasma cells are often spread out from the vessels into the adjacent grey and white matter and there is diffuse microglial hyperplasia, proliferation of large astrocytes, and formation of microglial nodules. A characteristic, but by no means pathognomonic, feature of trypanosomiasis is the morular (or Mott) cell, a modified plasma cell with a small, peripheral nucleus, and cytoplasm filled with globules of immunoglobulin (Russell bodies). These cells are present in the inflammatory infiltrate in the meninges and brain, but may also be found isolated in otherwise unaffected areas, especially in *T. b. rhodesiense* encephalitis, often associated with lymphophagocytosis. The diffuse reactive astrocytosis, is known to be a common event in this disease; once activated, astrocytes may express and secrete a number of cytokines. Conversely, a number of cytokines induce gliosis. Within the CNS trypanosoma is responsible for involving virtually all its components; injury to the choroid plexuses will enable the parasite to invade the CSF at a very early stage of the infection; parasites may reach the brain parenchyma through the Virchow-Robin spaces and this may represent the progression from meningitis to encephalitis. Parasite products can activate CD8+ T-cells, which secrete interferon-γ and interleukin-2 [43]; these activate macrophages to produce nitric oxide, tumour necrosis factor-α, and other products, which stimulate the astrogliotic reaction. Trypanosomes are rarely demonstrable in the histological sections and are only rarely seen in the meninges. Apparently, the differences between the encephalitis produced by the two organisms are exclusively quantitative and probably related to the stage at which the observations were made [2, 16].

5.2. American Trypanosomiasis (Chagas' Disease). Chagas' disease is caused by the protozoan *Trypanosoma cruzi*, which can take up the undulating, blood stream flagellate trypomastigote form, or the *Leishmania*-like amastigote form. Chagas' disease or American trypanosomiasis is widespread throughout Latin America, particularly in rural areas, but international migration is exporting the disease to developed countries [5, 38]. Night-biting triatomine bugs living on livestock or in cracks in walls are the vectors. Mature parasites are excreted in the faeces of the bugs whilst they feed, and inoculated directly through broken skin because of itching and scratching, or via transfer on fingers into the conjunctival sac.

Acute infection tends to be a mild self-limiting febrile illness. Approximately 30% of infected individuals develop chronic Chagas disease, which most commonly affects the heart (causing cardiomyopathy and dysrhythmias) or the digestive system (causing mega-oesophagus or mega-colon). Immune suppression from any cause may result in reactivation of latent infection, causing extensive necrotizing encephalitis [38, 44, 45].

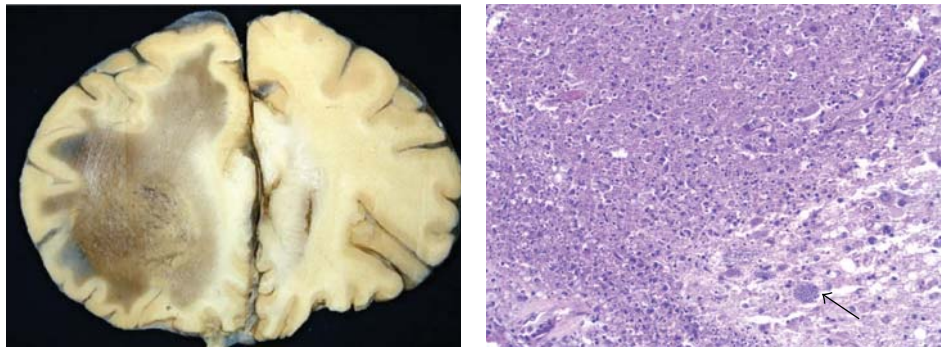
5.2.1. Neuropathology of CNS Lesions. CNS involvement may occur in the acute infective stage, not so clear in the chronic stage, and in reactivation of latent infection in the chronic phase in immunosuppressed patients. In the last context, new pathological presentations of the disease have been recorded in HIV-infected patients [45].

(1) Macroscopic Appearances. The changes are not conspicuous, except in the reactivated form, which will be described below. In the acute phase the brain appears swollen and congested, with scattered petechial hemorrhages. In the chronic phase there are usually no macroscopic changes.

(2) Microscopic Appearances. In the acute phase, there is a diffuse meningoencephalitis with multiple inflammatory foci, lymphocytes, some polymorphs, and macrophages throughout the CNS, with the tissue damage apparently due directly to destruction caused by the parasite after rupture of the cells. There are perivascular infiltrates of lymphocytes scattered within the brain parenchyma and amastigote (Leishmania-like) forms of the parasites within astroglial cells or less frequently at the center of microglial nodules, which are also scattered within the brain parenchyma, macrophages and endothelial cells. Lesions can also be seen in the meninges, and choroid plexus. Amastigote forms can be detected by conventional histology, by immunofluorescence and *in situ* hybridization.

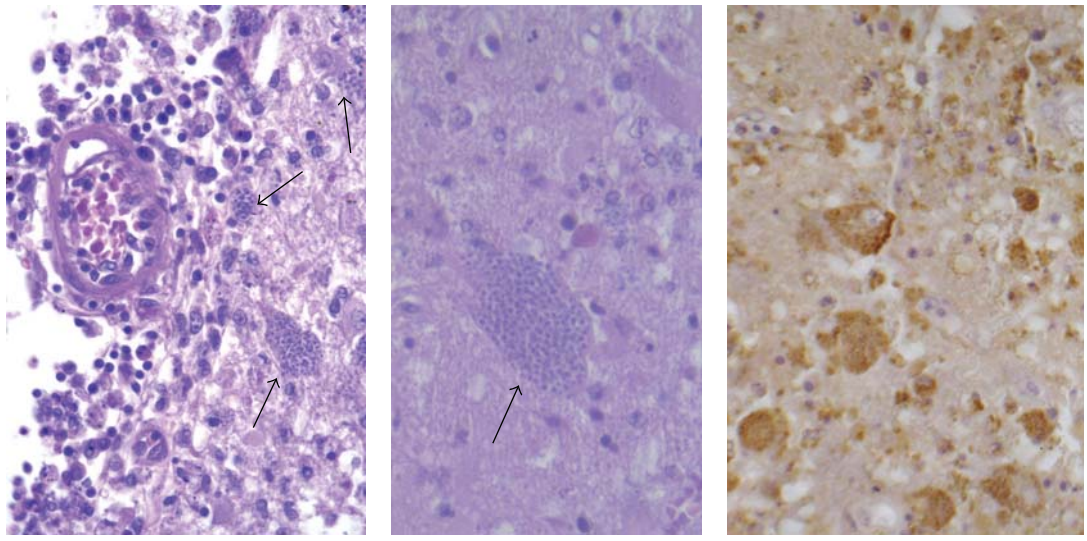
The existence of a chronic form affecting the CNS including a range of unexplained clinical presentations has not been well documented morphologically. Few small, hypocellular microglial nodules and aggregates of lymphoid cells sparsely distributed in the nervous tissue of some patients may be found, but no parasites, except in one case [46]. Neuronal loss is observed, but may be due to chronic hypoxia secondary to the cardiomyopathy accompanying chronic Chagas' disease. These relatively insignificant inflammatory changes are interpreted as being of a residual nature, possibly representing sequelae of the inflammatory nodules of the acute form, reinforcing the view against the existence of an anatomical basis for a chronic nervous form in Chagas' disease [2, 44, 45].

(3) Reactivated Disease. It occurs in patients chronically infected with *T. cruzi* who are immunosuppressed because of malignant neoplasms of the hematopoietic-lymphoid system, renal, heart, and bone marrow transplantation and especially because of HIV infection. It should be considered as a differential diagnosis of men-ingoencephalitis and space-occupying lesions in HIV patients with low CD4 T-cell



(a) (b)

Figure 13: Reactivated Chagas' disease. (a) An extensive acute necrotizing encephalitis seen histologically in (b), where amastigote forms of *T. cruzi* (arrow) are present, H&E.



(a) (b) (c)

Figure 14: Reactivated Chagas'disease. Lymphoplasmacytic leptomeningitis with subpial amastigote forms of the parasite (a, b) (arrows). The identity of the parasite was confirmed immunohistochemically (c) (a, b), H&E.

counts, living in or coming from an endemic area, especially if a mass lesion does not respond to therapy against toxoplasmosis [47–50].

Macroscopically, it takes the form of extensive necrotizing encephalitis and many patients have the pseudotumoral form, characterized by the presence of single or multiple necrotic-hemorrhagic nodular lesions, mostly located in the cerebral lobes and, in some cases, in the brain stem and cerebellum. The lesions are poorly limited, measuring several centimeters, and preferentially involve the white matter (Figure 13).

Histologically, this is an acute necrohaemorrhagic encephalitis characterized by microglial nodules, with associated haemorrhage, necrosis, and exudates of macrophages, lymphocytes, plasma cells and, to a lesser extent, neutrophilic granulocytes within the nervous tissue and perivascular spaces. Amastigote forms of the parasite are abundant within macrophages and astroglia but are not found in nerve cells. The identity of the parasite can be confirmed immunohistochemically. Lymphoplasmacytic leptomeningitis is a constant finding and apparently represents an extension of the subjacent necrotic-inflammatory lesions [2, 16, 45] (Figure 14).

(4) Other Causes of CNS Pathology. In chronic Chagas' disease, they include vascular associated lesions (ischemic or hemorrhagic) that are related to the chronic myocardiopathy that courses with congestive heart failure, dysrhythmias, and thromboembolic phenomena. These include secondary hypoxic nerve cell changes, cerebral infarcts, and hemorrhages [2, 45, 51, 52].

In addition, mild meningoencephalitis with epithelioid and giant cells may occur in cases of congenital Chagas' disease [53, 54]. The risk of infected mothers in the chronic phase, transmitting the disease to their fetus during pregnancy is however very low, probably <1% [55].

Components of the *peripheral nervous system* may also be affected in the acute phase of Chagas' disease. The autonomic tissue of the heart, oesophagus, and gut are especially susceptible, leading to the chronic cardiopathy with cardiomegaly, and the digestive megaviscera. The peripheral somatosensory neuropathies, including involvement of dorsal root ganglia, anterior horn neurons and peripheral sensory and motor nerve fibres are less frequent than the autonomic, but have been well documented clinically and electrophysiologically in chronic cases, supported by morphological reports in humans and experimental infection, showing both axonal and demyelinating neuropathies. They are postulated to result from several autoimmune mechanisms [2].

6. Microsporidiosis

Microsporidia are single-celled, obligate intracellular parasites. More than 20 genera of microsporidium are pathogenic in mammals, but *Encephalitozoon* species affect immunosuppressed populations more commonly than other species [1, 56]. *Trachipleistophora anthropophthera* may also cause encephalitis. In humans, microsporidium can be transmitted via contaminated water or air droplets and via the fecal-oral route. Sexual transmission of *Encephalitozoon* species may also occur.

The parasites have an internal coiled tube through which sporoplasm is extruded to pierce and inject infectious sporoplasm into the cytoplasm of the host cell. Disseminated infection with CNS involvement has been reported following kidney, pancreas, and bone marrow transplantation. Rare cases of CNS involvement have been reported in immunocompetent hosts [2].

6.1. Macroscopic Appearances. Microsporidiosis presents in the CNS as diffuse, nodular encephalitis, sometimes with necrotic foci. Multifocal lesions in gray or white matter can mimic cerebral toxoplasmosis.

Mild meningeal opacity has been reported.

6.2. Microscopic Appearances. There may be small foci of necrosis. The parasites may be detected in tissue biopsy specimens. Astrocytes are parasitized (but not neurons) and there is localized microglial proliferation. They are seen in clusters as haematoxyphilic nuclear dots within refractive clear cytoplasm, often Gram- and Ziehl-Neelsen-positive. Electron microscopy is more successful than light microscopy to identify the organisms [56]; molecular diagnosis with PCR of tissue biopsy samples is highly sensitive but is not widely available. During CNS infection, spores are often present in peripheral blood.

7. Leishmaniasis

While visceral leishmaniasis (*Leishmania donovani*) is a relatively common infection in all continents except Australasia, and spreads haematogenously in the body, CNS involvement is extremely rare [1].

Experimentally, *L. amazonensis* can cause encephalitis with parasites in the cerebral parenchyma [57] but there is only one recorded case of CNS infection by *L. donovani* in man. A child with drug-refractory visceral leishmaniasis had meningitis associated with parasites in the CSF.

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Research Article

Laminar Distribution of the Pathological Changes in Sporadic and Variant Creutzfeldt-Jakob Disease

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The laminar distributions of the pathological changes in the cerebral cortex were compared in the prion diseases sporadic Creutzfeldt-Jakob disease (sCJD) and variant CJD (vCJD). First, in some cortical regions, the vacuolation ("spongiform change") was more generally distributed across the cortex in sCJD. Second, there was greater neuronal loss in the upper cortex in vCJD and in the lower cortex in sCJD. Third, the "diffuse" and "florid" prion protein (PrP^{sc}) deposits were more frequently distributed in the upper cortex in vCJD and the "synaptic" deposits in the lower cortex in sCJD. Fourth, there was a significant gliosis mainly affecting the lower cortex of both disorders. The data suggest that the pattern of cortical degeneration is different in sCJD and vCJD which may reflect differences in aetiology and the subsequent spread of prion pathology within the brain.

1. Introduction

Four subtypes of the prion disease Creutzfeldt-Jakob disease (CJD) have been described to date including familial CJD (fCJD), linked to germline mutations of the prion protein (*PrP*) gene [1], and iatrogenic CJD (iCJD), a transmissible form of the disease in which the disease form of prion protein (PrP^{sc}) is acquired from growth hormone, dura mater grafts, or corneal transplantation [2]. By contrast, sporadic CJD (sCJD), the commonest form of the disease, may arise as a result of random mutation or posttranslational modification of the *PrP* gene [3, 4]. In addition, a new type of CJD, namely, variant CJD (vCJD), has been described in the United Kingdom [5]. Variant CJD differs significantly from previously described subtypes of the disease in having a younger age of onset (median age 23 years, range: 18–53 years), a prolonged duration of disease (12 to 24 months), a psychiatric presentation of the disease, and absence of typical EEG features [5]. Variant CJD has been linked to the consumption of meat originating from cattle with bovine spongiform encephalopathy (BSE) [5].

Neuropathologically, CJD is characterized by the presence in the brain of vacuolation "spongiform change", neuronal loss, a reactive astrocytosis, and the deposition of PrP^{sc} in the form of discrete deposits or plaques [6, 7]. There are different morphological types of PrP^{sc} deposit in CJD. Hence, "florid" deposits are composed of a central "core" surrounded by a rim of small vacuoles and are especially characteristic of vCJD. Broad bundles of amyloid are present within the core, and the deposits resemble more closely the "classic" deposits typical of Alzheimer's disease (AD) [8] rather than the "kuru"-type deposits characteristic of prion disease [9]. In addition, there are more "diffuse-type" deposits in vCJD (also known as "fine feathery diffuse deposits" or "fine diffuse plaques") [10] which are less aggregated, more weakly stained, and lack a distinct core [11]. By contrast, in the commonest type of sCJD, PrP^{sc} occurs in the form of "synaptic-type" deposits, and there are relatively few florid deposits or kuru plaques [12, 13].

Differences in pathology between subtypes of CJD could reflect variations in aetiology and the subsequent spread of prion pathology within the brain. Various hypotheses have been proposed to explain how PrP^{sc} spreads into the brain in CJD, for example, direct neural transmission from the site of infection, replication of PrP^{sc} in the spleen followed by neural entry through the spinal cord [14], infection of gut-associated lymphoid tissue with subsequent spread to the dorsal motor nucleus of the vagus nerve [15, 16], and

spread via the circulatory system [17]. In neurodegenerative disorders, such as AD, dementia with Lewy bodies (DLB), and Pick's disease (PiD), the density of the pathological changes within the cerebral cortex often varies significantly across the different cortical laminae [18–20]. Neocortical regions are characterized as having six laminae (I to VI), each of which has a specific pattern of connections. Moreover, the laminar distribution of a pathological change may reflect the degeneration of specific anatomical pathways which have their cells of origin or axon terminals within particular cortical laminae [21]. Hence, the present study compared the laminar distributions of the pathological changes in cases of sCJD and vCJD to determine whether the pattern of cortical degeneration was different in the two prion disorders.

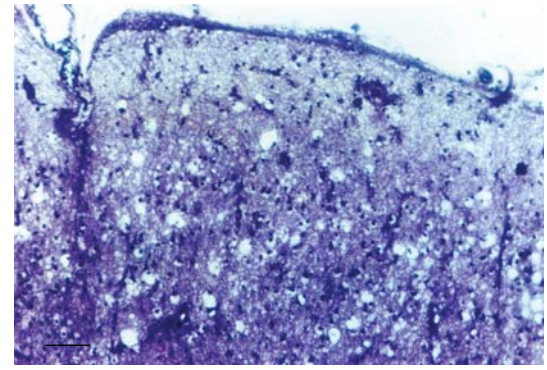

Figure 1: Vacuolation in the upper cortical laminae of the frontal cortex in a case of sporadic Creutzfeldt-Jakob disease (sCJD). (cresyl violet, magnification bar = 200 μm).

2. Materials and Methods

2.1. Cases. Eleven cases each of sCJD and vCJD (details in Table 1) were studied at the Brain Bank, Department of Neuropathology, Institute of Psychiatry (IOP), King's College London, UK. Informed consent was obtained for the removal of all brain material according to the 1999 Declaration of Helsinki (as modified Edinburgh 2000). Brain material of sCJD was obtained from the IOP and of vCJD from the National CJD Surveillance Centre, Western General Hospital, Edinburgh, UK. All cases fulfilled the neuropathological diagnostic criteria for CJD [22]. None of the cases had any of the known mutations of the *PrP* gene or family history of prion disease, and there was no evidence of the known types of iatrogenic aetiology. In the vCJD cases, the pattern of PrP^{sc} deposition typical of these cases was observed with florid-type PrP^{sc} deposits in the cerebral cortex, cerebellum, basal ganglia, thalamus, and brain stem [23, 24]. In addition, sCJD is classified according to heterogeneity at the polymorphic codon 129 of the *PrP* gene and the presence of Type 1 or Type 2 isoforms of PrP^{sc} [13]. The present cases conformed to the commonest subtype of sCJD, that is, homozygous for methionine at codon 129 and with Type 1 PrP^{sc} (M/M1). All vCJD cases studied were methionine/methionine (M/M) homozygotes at codon 129. In addition, the PrP^{sc} characteristic of vCJD had a uniform glycotype (PrP^{sc} Type 4) [23].

2.2. Histological Methods. Blocks of the frontal cortex (B8) at the level of the genu of the corpus callosum, parietal cortex (B7) at the level of the splenium of the corpus callosum, occipital cortex including the calcarine sulcus, inferior temporal gyrus (B22), and parahippocampal gyrus (B28) were taken from each case. Tissue was fixed in 10% phosphate-buffered formal saline and embedded in paraffin wax. Sequential, coronal 7 μm sections were stained with haematoxylin and eosin (H/E), cresyl violet, or immunostained against PrP using the monoclonal antibody 12F10 (dilution 1 : 250) which binds to a region of human PrP downstream of the neurotoxic domain adjacent to helix region 2: residues 142–160 [25] (kindly provided by Prof. G. Hunsmann, The German Primate Centre, Gottingen, Germany). Immunoreactivity was enhanced by formic acid (98% for 5 minutes) and autoclaving (121°C for 10 minutes) pretreatment. Sections were treated with Dako Biotinylated Rabbit anti-Mouse (RAM) (dilution 1 : 100) and Dako ABComplex HRP kit for 45 minutes (Amersham, UK). Diaminobenzidine tetrahydrochloride was used as the chromogen. Immunostained sections were counterstained with haematoxylin for 1 minute to reveal neuronal cell bodies and glial cell nuclei.

2.3. Morphometric Methods. The distribution of the vacuolation, surviving neurons, glial cell nuclei, and PrP^{sc} deposits across the cortical lamainae was studied using methods similar to those of Duyckaerts et al. [26]. Five traverses from the pia mater to the edge of the white matter were located at random within each cortical area. With the exception of the synaptic PrP^{sc} deposits in sCJD, all pathological changes were counted in $50 \times 250\,\mu$m sample fields, the larger dimension of the field being located parallel with the surface of the pia mater. An eyepiece micrometer was used as the sample field and was moved down each traverse one step at a time from the pia mater to white matter. Histological features of the section were used to correctly position the field. Counts from the five traverses were added together to study the distribution of the pathology within each cortical region. Because of the diffuse nature of the synaptic deposits in sCJD, these lesions were quantified by "lattice sampling", that is, by counting the number of times the intersections of the grid lines encountered a PrP^{sc} deposit [27].

2.4. Data Analysis. No attempt was made to locate precisely the boundaries between individual cortical laminae. First, the degree of pathological change and cell losses in the CJD cases made laminar identification difficult. Second, identification is especially difficult in the frontal cortex because it exhibits a heterotypical structure, that is, six laminae cannot be clearly identified even when the cortex is fully developed. Instead, variations in lesion density with distance below the pia mater were analysed using a curve-fitting procedure (STATISTICA software, Statsoft Inc., 2300 East 14th St, Tulsa, OK 74104, USA) [28, 29]. Hence, for each cortical region, a linear, quadratic, cubic, and quartic polynomial was fitted

Table 1: Demographic data and gross pathological features of the sporadic (sCJD) and variant Creutzfeldt-Jakob disease (vCJD) cases studied.

Case	Sex	Age at onset (years)	Duration (years)	Brain weight (gm)	Gross atrophy
sCJD					
A	F	71	0.25	1222	Bilateral, diffuse
B	F	59	2.4	986	Severe Fr, T, C
C	F	71	0.16	1275	Mild Fr
D	F	71	0.25	1169	Mild
E	M	78	0.25	1562	Moderate
F	M	50	0.25	1292	None
G	M	67	0.16	1425	Moderate
H	F	69	0.16	1365	Mild
I	M	60	NA	1621	None
J	M	61	1	1270	Mild
K	F	78	0.40	1061	Mild, diffuse
vCJD					
A	F	39	2	586 L	None
B	F	28	1	1375	None
C	F	28	1	NA	NA
D	M	19	1	NA	NA
E	M	30	1	699 R	None
F	M	48	2	1470	None
G	F	34	1	810 L	None
H	M	18	1	1434	None
I	M	24	1	NA	NA
J	F	21	2	1394	None
K	M	35	1	718 R	None

Abbreviations: M: Male, F: Female, Fr: Frontal cortex, T: temporal cortex, C: cerebellum, R: Right hemisphere, L: Left hemisphere, NA = data not available.

Table 2: Frequency of particular types of distribution of the vacuolation, surviving neurons, glial cell nuclei and prion protein (PrP^{sc}) deposition across the cortical laminae in the cerebral cortex of cases of sporadic Creutzfeldt-Jakob disease (sCJD) and variant CJD (vCJD).

		Type of distribution								
					Unimodal			Bimodal		
Feature	Case	*N*	NS	RD	*U*	*M*	*L*	*U* > *L*	*U* = *L*	*U* < *L*
Vacuoles	sCJD	43	6	10	3	0	4	7	8	5
	vCJD	58	0	17	9	1	9	7	14	1
Neurons	sCJD	43	4	5	19	0	0	12	3	0
	vCJD	52	12	11	8	0	3	6	10	2
Glia cells	sCJD	42	1	1	0	0	37	0	1	2
	vCJD	57	3	3	2	2	44	0	2	1
PrP^{sc}	sCJD	31	4	3	2	0	13	4	2	3
Diffuse PrP^{sc}	vCJD	55	10	3	21	0	7	6	6	1
Florid PrP^{sc}	vCJD	51	14	1	16	1	6	6	6	1

N: number of neocortical regions studied, NS: no significant difference in density with laminar depth, RD: restrictive distribution, sparing the superficial laminae and the region adjacent to white matter, U: upper cortical laminae, M: middle cortical laminae, L: lower laminae.

successively to the data. At each stage, the goodness of fit of the polynomial to the data was tested using correlation methods and analysis of variance. A more complex curve was accepted as a better fit to the data if it resulted in a significant increase in Pearson's correlation coefficient ("*r*") and a significant reduction in the residual sums of squares compared with the preceding curves [28]. The distribution of the pathological changes in each cortical area was then classified according to whether a single (unimodal) or double (bimodal) peak of density was present (Table 2). If the distribution was unimodal, a further classification was made according to whether the density peak was located in

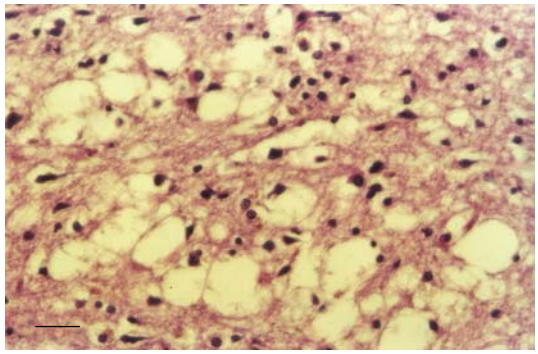

Figure 2: Vacuolation and gliosis in the occipital cortex in a case of sporadic Creutzfeldt-Jakob disease (sCJD), (H/E, magnification bar = 50 μm).

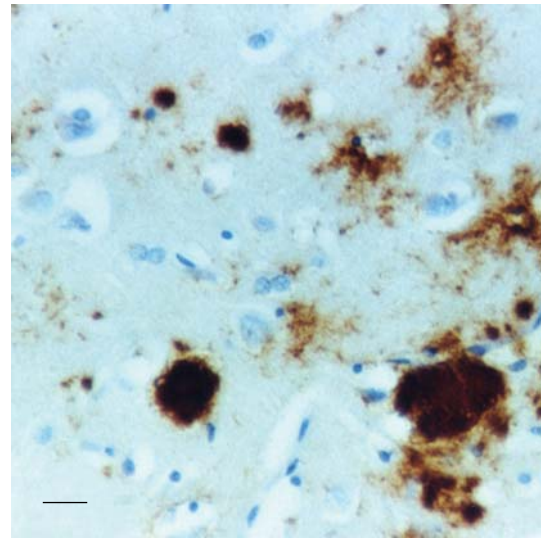

Figure 3: Prion protein (PrP^{sc}) deposits in the frontal cortex of a case of variant Creutzfeldt-Jakob disease (vCJD), (PrP^{sc} immunohistochemistry, magnification bar = 50 μm).

the upper laminae, in the middle of the profile, or in the lower laminae. If a bimodal distribution was present, the data were further classified according to whether the upper density peak was greater than, equal to, or less than the lower density peak. There were several regions in which a pathological feature was distributed across the cortical laminae but also there were spared regions close to the pia mater and adjacent to the white matter. Differences in the frequency of the different patterns of distribution of each histological feature in the sCJD and vCJD cases were statistically tested using chi-square (χ^2) contingency table tests.

3. Results

Figure 1 shows a low-power section of the frontal cortex of a case of sCJD showing the vacuolation affecting all laminae but especially the superficial cortical laminae. In Figure 2, a high-power section shows the vacuolation and gliosis in the lower laminae of the occipital cortex in a case of sCJD. Figure 3 shows a typical section of the frontal cortex in a case of vCJD immunolabeled with antibodies raised against PrP^{sc} and reveals the strongly immunolabeled florid PrP^{sc} deposits which are composed of a condensed core of PrP^{sc} and the more lightly immunolabeled and irregularly shaped diffuse deposits.

Typical examples of the laminar distribution of the PrP^{sc} deposits in the CJD cases studied are shown in Figure 4. In vCJD (Case A, occipital cortex), the distribution of the diffuse PrP^{sc} deposits was fitted by a first-order polynomial ($r = 0.82$, $P < .001$) suggesting a linear decline in abundance with distance below the pia mater. By contrast, in sCJD (Case C, Parietal cortex), the distribution of the synaptic PrP^{sc} deposits was fitted by a second-order (quadratic) polynomial ($r = 0.81$, $P < .001$) suggesting a peak of abundance in the lower cortical laminae.

A summary of the laminar distributions of the pathological changes in all brain areas is shown in Table 2. First, the distribution of the vacuolation in sCJD and vCJD was similar in many brain areas. Nevertheless, in sCJD there were a higher proportion of brain areas exhibiting

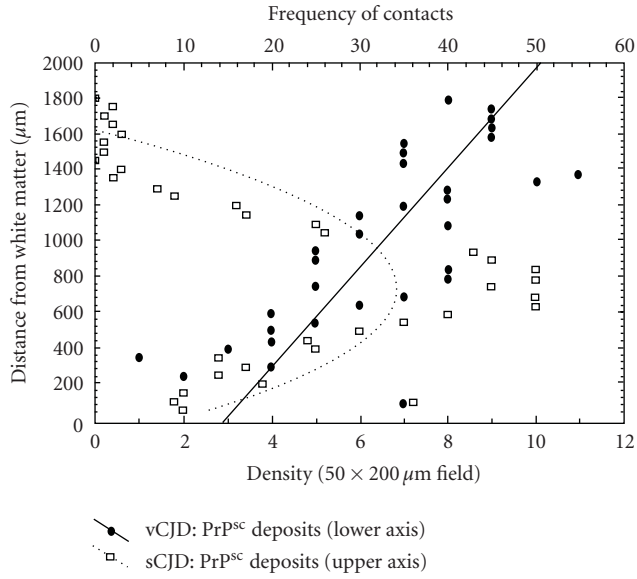

Figure 4: Distribution of the prion protein (PrP^{sc}) deposits with distance across the cortical laminae in the cerebral cortex Creutzfeldt-Jakob disease (CJD). Diffuse deposits in variant CJD (vCJD), fit to polynomial (linear $r = 0.82$, $P < .001$); PrP^{sc} deposits in sporadic (CJD) (sCJD), fit to polynomial (second-order $r = 0.81$, $P < .001$).

vacuolation across all cortical laminae whereas in vCJD, there were a higher proportion of areas in which the vacuolation spared the superficial laminae and the region adjacent to the white matter. Second, in vCJD, the surviving neurons were more frequently evenly distributed across the cortex or exhibited a bimodal distribution in which the size of the upper and lower peaks was similar. By contrast in sCJD, in

regions where a bimodal distribution of surviving neurons was present, the density peaks were asymmetric, the larger density peak occurring in the upper laminae. Third, the laminar distribution of the glial cell nuclei was similar in both sCJD and vCJD, the gliosis being most prominent in the lower cortex. Fourth, there were significant differences in the distribution of the PrP^{sc} deposits in sCJD and vCJD. The diffuse and florid PrP^{sc} deposits were more frequently distributed in the upper cortex in vCJD while the synaptic deposits were predominantly distributed in the lower cortex in sCJD. There were no essential differences in the laminar distribution of histological features in allocortical areas, for example, PHG and isocortical areas in either sCJD or vCJD.

4. Discussion

There were similarities and differences in the laminar distributions of the histological and pathological changes in sCJD and vCJD. First, there were similarities in the distribution of the vacuoles in the two disorders, but in sCJD, there were more brain regions in which the vacuoles were distributed across all laminae while in vCJD, there were more regions in which the vacuolation spared the superficial laminae and the region immediately adjacent to the white matter. Hence, the vacuolation may be more extensively distributed across the laminae in more regions in sCJD than in vCJD.

Second, there was a marked difference in the distribution of the surviving neurons in sCJD and vCJD. In normal control brain, cortical neurons are often bimodally distributed with peaks of density in the upper and lower cortex, the two peaks being asymmetric and the upper density peak being significantly larger than the lower density peak [18–20]. In sCJD, however, there were an increased number of brain regions in which the surviving neurons were present at a greater density in either the upper cortex alone or a bimodal distribution was present in which the density peak in the upper cortex was larger than in the lower cortex. In vCJD, surviving neurons were more frequently either uniformly distributed or in a bimodal distribution with equal-sized peaks. These results suggest greater neuronal loss in the upper cortex in vCJD and in the lower cortex in sCJD.

Comparisons between sCJD and vCJD (χ^2 contingency tables): (a) All categories including totals for the bimodal distributions: Vacuoles $\chi^2 = 11.87$ (5DF, $P < .05$); Neurons $\chi^2 = 13.27$ (4DF, $P < .05$), Glial cells $\chi^2 = 4.43$ ($P > .05$); Diffuse PrP^{sc} versus Synaptic $PrP^{sc}\chi^2 = 15.72$ (4DF, $P < .01$), Florid PrP^{sc} versus Synaptic $PrP^{sc}\chi^2 = 17.94$ (5DF, $P < .001$); (b) comparison of unimodal distributions only: Vacuoles $\chi^2 = 0.48$ (2DF, $P > .05$); Neurons $\chi^2 = 3.12$ (1DF, $P > .05$), Glial cells $\chi^2 = 3.23$ (2DF, $P > .05$); Diffuse PrP^{sc} versus Synaptic $PrP^{sc}\chi^2 = 12.55$ (1DF, $P < .01$), Florid PrP^{sc} versus Synaptic $PrP^{sc}\chi^2 = 13.37$ (2DF, $P < .01$); (c) comparison of bimodal distributions only: Vacuoles $\chi^2 = 4.21$ (2DF, $P > .05$), Neurons $\chi^2 = 7.56$ (2DF, $P < .05$), Glia cell nuclei $\chi^2 = 0.67$ (2DF, $P > .05$), Nonflorid PrP^{sc} χ^2 versus Synaptic $PrP^{sc}\chi^2 = 2.76$ (2DF, $P > .05$), Florid PrP^{sc} versus Synaptic $PrP^{sc}\chi^2 = 2.76$ (2DF, $P > .05$).

Third, there are similarities in the distribution of glial cell nuclei in sCJD and vCJD which showed a marked preference for the lower cortical laminae, a distribution also reported in previous studies [30]. Although this distribution could reflect greater pathological change in the lower cortex in sCJD, where it is accompanied by greater neuronal loss and PrP^{sc} deposition, this is unlikely to be the case in vCJD where neuronal losses appear to be greater in the upper cortical laminae. It is possible that in sCJD, the glial cell reaction directly reflects pathological changes in the lower laminae but in vCJD reflects greater degeneration of the afferent and efferent subcortical pathways which have their cells of origin or axon terminals in the lower cortex.

Fourth, significant differences in the laminar distribution of PrP^{sc} deposits were observed in the two disorders. In vCJD, the diffuse and florid PrP^{sc} deposits showed a marked preference for the upper cortical laminae while the synaptic deposits of sCJD were more frequently distributed in the lower cortex. These distributions could be related to the degeneration of different anatomical pathways in the neocortex. Hence, the distribution of the pathological changes in vCJD suggests degeneration of the feed-forward corticocortical projections while degeneration of the feedback corticocortical pathways and/or the feedback pathways may be present in sCJD [21, 31].

Differences in the distribution of the pathology in sCJD and vCJD could reflect the differences in the aetiology, and subsequent spread of prion pathology. Hence in vCJD, prions are believed to enter the nervous system by absorption through the gut following consumption of infected food, replication in the spleen, and neural entry via the spinal cord [14] whereas sporadic cases are less likely to have an iatrogenic aetiology, and to result from either random mutation or posttranslational modification of the *PrP* gene [3, 4]. The different origins of the pathogenic prions may affect the subsequent development and spread of the pathology through the brain. In vCJD, for example, the pathology may spread into the cerebellum from the spinal cord via the anterior spinocerebellar tract [32]. Subsequent spread may then involve the loop of connections involving the thalamus, neocortex (especially the feed-forward connections), pons, and cerebellum [33]. By contrast, in sCJD, the pathological prions may be formed *in situ,* and the distribution of the pathology will reflect the site of origin and the pathways by which prion pathology spreads from these sites. Hence, if there are multiple sites of origin of the pathology, there may be a less marked topographic pattern to the pathology in sCJD than in vCJD.

5. Conclusions

The data are consistent with the hypothesis that there are different patterns of cortical degeneration in sCJD and vCJD. All laminae of the neocortex may be affected in both sCJD and vCJD, but, there is a greater development of the pathology in the lower laminae in sCJD and in the upper laminae in vCJD. Differences in cortical degeneration may be directly related to the different aetiologies of the two

disorders and differences in the pattern of spread of the pathology through the brain.

Conflict of Interests

The author reports no conflicts of interests.

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