Citrobacter diversus brain abscess: case reports and molecular epidemiology

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Summary. *Citrobacter diversus* brain abscess occurred in two infants in Aberdeen, 5 months apart. These are the first reported cases of this condition in the UK since 1976. Restriction endonuclease analysis with *SacI* enzyme showed blood and CSF isolates from both patients to be identical and different from 10 other clinical isolates of *C. diversus* and one *C. amalonaticus* strain. Furthermore, isolates of *C. diversus* from patients belonged to biotype "d" whereas control isolates were of biotypes "a" or "e". Because both infants attended the same central and peripheral maternity units, this raised suspicions of long term contamination of the hospital environment by this organism. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of whole-cell proteins and immunoblotting with normal human serum were remarkably homogeneous for all 13 *C. diversus* strains and thus were not useful for typing. However, the only *C. amalonaticus* strain was clearly differentiated from *C. diversus* strains by SDS-PAGE. Management of the infants included multiple intravenous antibiotic therapy for 4–6 weeks and repeated computerised tomography (CT) scanning and drainage of the abscess cavity. Both children survived albeit with some minor degree of brain damage.

Introduction

Citrobacter diversus (syn. *C. koseri* or *Levinea malonatica*) is now considered to be the organism most frequently associated with neonatal brain abscess.¹ Other members of the family Enterobacteriaceae, e.g., *Escherichia coli*, are more commonly associated with neonatal meningitis. However, *C. diversus* meningitis is very often complicated by brain abscess formation (77%).^{2,3} Furthermore, the mortality rate is high (34%) and the majority of children who recover suffer varying degrees of brain damage;³ <5% of infants make a complete recovery. Since the first reports of nursery outbreaks of *C. diversus* meningitis from England in 1973,^{4,5} several sporadic cases^{1,6,7} as well as small outbreaks^{8–11} have been reported from the USA. The last report from the UK was that of Ribeiro *et al.*¹² who described an outbreak of neonatal *C. diversus* meningitis in a Manchester hospital in 1976.

The epidemiology of *C. diversus* neonatal meningitis remains unclear, largely because a satisfactory typing system does not exist. Biotyping,^{8–10} antibiograms^{9,10} and serotyping^{8,10,11} have been used to characterise strains. However, organisms associated with out-

breaks of meningitis belong to several biotypes and serotypes and correlation between these various markers is poor.¹¹ Kline *et al.*¹³ have found that *C. diversus* strains causing neonatal meningitis are more likely than others to possess a 32-Kda outer-membrane protein (OMP) when examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). In a further study,¹⁴ strains possessing the 32-Kda OMP were found to be genetically heterogeneous by multilocus enzyme electrophoresis. Restriction endonuclease analysis (REA) with multiple enzymes has been used in the investigation of meningitis outbreaks in three Maryland hospitals.¹¹ In two of these hospitals, identical *C. diversus* strains were isolated from affected infants, healthy neonates and nursery personnel. Different *C. diversus* strains were also isolated in all three hospitals.

We report here the first two cases of *C. diversus* brain abscess recorded in the UK since 1976. Three isolates of *C. diversus* from CSF and blood of these patients were compared to 11 other clinical isolates with respect to biotype, antibiogram, SDS-PAGE of whole-cell proteins (WCP) and immunoblotting with normal human serum (NHS); REA was also performed on all strains. SDS-PAGE of WCP and immunoblotting have not previously been reported for *C. diversus* strains associated with meningitis and brain abscess.

Case reports

Case 1

A female infant was delivered at term by elective caesarean section because of cephalopelvic disproportion. She appeared well at birth, was moved on the second day from the central maternity hospital to a peripheral unit, and was discharged home well on day 10. She was admitted to the Children's Hospital 24 h later because she was febrile and reluctant to feed; examination revealed an ill, irritable, pale infant but no specific abnormal findings. A full infection screen was performed and the cerebrospinal fluid (CSF) was found to be turbid with a white cell count of $1.97 \times 10^9/L$, 94% of which were polymorphonuclear leucocytes. Gram-negative bacilli were seen in the gram-stained film. Treatment was started with intravenous (i.v.) cefotaxime and gentamicin, and phenobarbitone was given because of the baby's extreme irritability. Ultrasound scan of her head 24 h after admission was reported as normal. No organisms were seen in CSF taken 24 h and 48 h after admission and the samples were sterile. At this point, the organism in the initial CSF specimen was identified as *C. diversus* whereupon chloramphenicol was added to the antibiotic regimen. The same species was identified in blood cultures taken on admission.

CT scan 1 week after admission demonstrated an abscess cavity 3 cm in diameter in the right frontal lobe, and several other suspicious areas of oedema. Frequent imaging by both ultrasound and CT scan demonstrated the evolution of two further abscess cavities, one in the left frontal lobe and the other in the left posterior parietal region. These cystic cavities were drained on several occasions and gentamicin was instilled. Very scanty gram-negative organisms were seen in the fluid on the first occasion, but there was no growth on culture. Progressive ventricular dilatation was controlled after 3 weeks by the insertion of a ventriculo-peritoneal (VP) shunt. The post-operative course was complicated by a cerebral haemorrhage in the region of the third ventricle and the infant developed seizures which were difficult to control. Intravenous antibiotics were continued for 6 weeks, although chloramphenicol had been stopped after 3 weeks. The baby made a slow recovery and at the time of discharge, 7 weeks after admission, she was markedly hypotonic, had poor visual responsiveness and her visual evoked responses (VERs) were abnormal.

At the age of 1 year, the child had nystagmus affecting her right eye only but her visual responsiveness and VERs were markedly improved. Her seizures were well controlled by anticonvulsant therapy. She was pulling to stand and cruising around the furniture; fine motor skills were generally a little delayed, but on the whole her progress was pleasing. A CT scan at that time showed a persisting right frontal cavity but the other cavities had completely resolved.

Case 2

Five months after Case 1, a female infant was delivered at term by emergency caesarean section because of failure of labour to progress. She was delivered in the same central maternity unit, transferred to the same peripheral unit after 48 h, and was discharged home well on day 10. She was admitted to the Children's Hospital at the age of 5 weeks with a 4-day history of vomiting, lethargy, reluctance to feed and constipation; on examination she appeared unwell but was afebrile. Her fontanelle was tense and there was marked splaying of the cranial sutures. Lumbar puncture was considered inadvisable and she was given intravenous ampicillin and chloramphenicol empirically. A CT scan demonstrated a 5-cm abscess cavity in the right frontal lobe. This cavity was aspirated and *C. diversus* was isolated from the fluid. Blood and stool culture results were negative. When the sensitivity pattern of the organism was known, the antibiotic regimen was changed to i.v. cefotaxime and gentamicin which were continued for 6 weeks. Intracranial events were monitored by head circumference measurements and frequent imaging by both CT and ultrasound scan. The abscess cavity was aspirated and gentamicin was instilled on a further six occasions. The infant developed seizures and a progressive communicating hydrocephalus which was controlled by VP shunt 5 weeks after the onset of her illness. Her post-operative course was uneventful and she was discharged 8 weeks after admission. At this time she was generally hypotonic, visually unresponsive and her VERs were of reduced amplitude and increased latency.

At 1 year old, the seizures were well controlled by anticonvulsant therapy and she was visually and socially responsive. Repeat VERs were much improved, although not normal. Her tone was normal and development seemed satisfactory although her fine motor skills were mildly delayed. A CT scan showed a large area of scarring in the left frontal region with some dilatation of the aqueduct and fourth ventricle.

Materials and methods

Samples

Three isolates of *C. diversus* (A-C) were obtained from CSF and blood of the two cases. Eleven other clinical isolates (D-N) were randomly selected from clinical material other than CSF or blood (table). These latter isolates were identified by the API 20E system (API Laboratories, Basingstoke, Hants) as *C. diversus/amalonaticus* and were not chosen on the basis of clinical significance. Swabs were also obtained from the wet environment of the labour ward, and the central and peripheral maternity units 9 months after the second case to determine whether there was contamination with *C. diversus*.

Biotyping and antibiograms

The API 20E result does not distinguish *C. diversus* from the closely related species *C. amalonaticus*. To make this distinction, extra biochemical tests were employed. Isolates classified as *C. diversus* gave positive results in tests for malonate utilisation, adonitol and D-arabitol fermentation whereas *C. amalonaticus* isolates gave negative results. Biotyping of *C. diversus* isolates was based on fermentation tests with dulcitol, sucrose, D-sorbitol and L-rhamnose.¹⁵ Antibiotic sensitivity tests were performed by a modification of the Stokes's disk diffusion method: with a rotary plater, the test organism was inoculated on the inside and the control organism (*E. coli* NCTC 10418) on the outside of DST (Oxoid) agar plates. Antibiotics tested with Oxoid sensitivity disks were ampicillin (10 µg), co-amoxycylav (30 µg), piperacillin (75 µg), cefuroxime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), trimethoprim (2.5 µg), gentamicin (10 µg), netilmicin (30 µg), amikacin (30 µg), ciprofloxacin (1 µg), imipenem (10 µg) and chloramphenicol (30 µg).

SDS-PAGE and immunoblotting

These were performed broadly in accordance with the method of Maggs and Pennington¹⁶ except as detailed below. All isolates were cultured on nutrient agar for 24 h at 37°C, and then harvested, washed, and resuspended in sterile saline before sonication, with cooling in an ice bath, for 2 min. Sonicated extracts were stored at -70°C until use. For SDS-PAGE and immunoblotting, sonicated extracts were mixed with three volumes of 0.01 M Tris-HCl buffer, pH 6.8, containing SDS 4.4% w/v, glycerol 20% v/v, 2-mercaptoethanol 20% v/v and bromophenol blue 0.001% w/v, and heated for 5 min in a boiling water bath. Samples were then centrifuged for 5 min at 11 500 g in a microcentrifuge, electrophoresed on an

acrylamide 10% separating gel with a 4% stacking gel and stained with Coomassie Brilliant Blue R.

Immunoblotting was performed by transferring by overnight electrophoresis material from acrylamide gels to nitrocellulose membranes (Hybond-C, Amersham, Bucks). The membranes were then incubated for 1 h at 37°C in a blocking buffer of 0.1 M phosphate-buffered saline, pH 7.4, containing skimmed milk powder (Oxoid) 3% w/v. Pooled normal human serum (Blood Transfusion Service, Aberdeen Royal Infirmary), diluted 100-fold in blocking buffer, was used as the primary antibody and peroxidase-labelled anti-human IgG (SAPU, Law Hospital, Carlisle), diluted 100-fold in blocking buffer, was used to detect antibody binding. Banding patterns obtained for different isolates were compared visually.

Restriction endonuclease analysis

DNA was extracted by a procedure based on that of Bjorvatn *et al.*¹⁷ Total yields, measured at 260 nm, were 0.5–4 mg/ml. Eleven restriction enzymes (*Apa*I, *Alu*I, *Bam*HI, *Dra*II, *Eco*RV, *Hae*III, *Kpn*I, *Pst*I, *Sac*I, *Sal*I and *Taq*I) were used to establish the most discriminatory banding patterns. *Sac*I gave the most useful banding pattern and was subsequently used for all further digests. DNA (5 µg) was digested overnight with 5 units of restriction endonuclease and electrophoresed in agarose 0.8% at 5 V/cm. DNA banding patterns were visualised under UV light after staining with ethidium bromide.

Results

Biotyping and antibiograms

The API profiles and biotypes of the 14 isolates are outlined in the table. One isolate (J) gave negative results in tests for utilisation of malonate and citrate

Table . *Citrobacter* strains and biotypes

Isolate designation	Source	API profile	Fermentation of				Biotype
			dulcitol	sucrose	D-sorbitol	L-rhamnose	
A	Blood (case 1)	134 4533	+	+	+	+	d
B	CSF (case 1)	134 4533	+	+	+	+	d
C	CSF (case 2)	134 4533	+	+	+	+	d
D	Stool	134 4513	—	—	+	+	a
E	CSU	134 4513	—	—	+	+	a
F	Sputum	334 4533	—	+	+	+	e
G	Sputum	334 4513	—	—	+	+	a
H	CSU	334 4513	—	—	+	+	a
I	CSU	334 4513	—	—	+	+	a
J	MSU	334 4513	—	—	+	+	<i>C. amalonaticus</i>
K	MSU	134 4513	—	—	+	+	a
L	MSU	334 4512	—	—	+	+	a
M	Burn graft	334 4513	—	—	+	+	a
N	Breast ulcer	334 4513	—	—	+	+	a

CSF, cerebrospinal fluid; CSU, catheter specimen of urine; MSU, midstream specimen of urine.

Fig. 1. SDS-PAGE of WCPs from 14 *Citrobacter* isolates. Lane A, case 1 blood isolate; B, case 1 CSF isolate; C, case 2 CSF isolate; D-I and K-N, control *C. diversus* isolates; J, *C. amalonaticus* isolate; mol.-wt markers (Kda) are between lanes G and H and listed.

Fig. 2. Immunoblot of 14 *Citrobacter* isolates with normal human serum. Lane designations correspond to those in fig. 1 and the table.

and for fermentation of D-arabitol and adonitol; therefore it was identified as *C. amalonaticus*. Of the 13 *C. diversus* strains, the three CSF and blood isolates from our patients (isolates A-C) were biotype "d". The remaining isolates were biotype "a" with the exception of isolate F which was biotype "e". All isolates were resistant to ampicillin and sensitive to the other antibiotics tested. Environmental sampling yielded no *C. diversus* isolates.

SDS-PAGE and immunoblotting

SDS-PAGE profiles of whole cell proteins showed remarkable homogeneity among *C. diversus* isolates

and there were no discernible differences between abscess-causing isolates and others. However, the only *C. amalonaticus* isolate (J) was clearly different (fig. 1). Immunoblotting with normal human serum revealed two prominent bands of high mol. wt (>66 Kda) but again provided no discrimination between *C. diversus* isolates. However, the *C. amalonaticus* strain was different, having only one prominent, high mol.-wt band (fig. 2).

Restriction endonuclease analysis

Fig. 3 shows the REA "fingerprints" of isolates A-N. Isolates A, B and C gave indistinguishable banding

Fig. 3. Agarose gel electrophoresis of *SacI* digests of whole-cell DNA extracted from 14 *Citrobacter* isolates. Lanes 1, 9 and 17, 1-kb markers; 2-8 and 10-16, *Citrobacter* isolates A-G and H-N respectively, designated as in fig. 1 and the table.

patterns. Isolates D-N gave unique banding patterns that were easily distinguishable from one another and from those of isolates A-C.

Discussion

Organisms belonging to the genus *Citrobacter* are widespread in the environment, being found in soil and water. *C. freundii*, the type species, is commonly carried in the large intestine and may be confused biochemically with *Salmonella* spp. (many strains are lactose non-fermenters and H₂S positive). On the other hand, the carriage rate of *C. diversus* in the intestine is thought to be low¹⁸ except during nursery outbreaks when it may be as high as 79%.^{8,12} *Citrobacter* spp. have been associated with significant nosocomial infection—including urinary tract infection, especially in catheterised patients, respiratory tract infections, bacteraemia and endocarditis, especially in elderly or debilitated patients. Most clinical isolates represent secondary infection or asymptomatic colonisation. However, *C. diversus* is now recognised as an important cause of neonatal meningitis² and the commonest cause of brain abscess in neonates.¹ Most affected infants are under 6 weeks of age and no cases have been recorded beyond 8 weeks. This suggests that host susceptibility is an important predisposing factor.

The pathogenesis of *C. diversus* meningitis and

brain abscess remains unclear. Studies in the infant rat¹⁹ have shown that the infection is haematogenous in origin. Leptomeningitis is followed by ventriculitis and direct extension of organisms into the adjacent brain tissue. *C. diversus* strains isolated from CSF did not differ from those isolated elsewhere with respect to pilation, haemolysin production and resistance to the killing effect of serum.¹³ One study demonstrated that *C. diversus* strains causing neonatal meningitis were more likely than others to possess a 32-Kda OMP.¹³ However, a further study by the same group²⁰ failed to "shed new light" on this association.

Our two cases had several features in common. They came from the same small town and shared the same General Practitioner and Health Visitor. Moreover, both babies were delivered at term by lower segment caesarian section and both were in the same central and peripheral maternity units in the immediate postnatal period. Although nearly 5 months elapsed between the first and second case, environmental transmission could not be excluded. Other workers have shown that *C. diversus* is capable of prolonged survival (up to 4 years) in the hospital environment,³ leading to protracted nursery outbreaks of meningitis and brain abscess, albeit with a low case:carrier ratio of 4%.^{8,12} A faecal reservoir of infection⁸ and transmission via hands⁹ of nursery personnel have been implicated in outbreaks. Vertical transmission of *C. diversus* has also been described,⁷ the organism being carried in the intestine and transmitted to the

infant during labour or in the immediate post-natal period. Considering the early onset of brain abscess formation in case 1 (9 days), vertical transmission is a real possibility, whereas case 2, with later onset disease (5 weeks), is more likely to have acquired the infection from the environment or from hospital staff. It was considered inadvisable to attempt to demonstrate faecal carriage of *C. diversus* in the mother because such knowledge might have proved distressing to already stressed parents. Furthermore, environmental contamination, staff carriage and infant colonisation with *C. diversus* were not investigated at the time because cross contamination was not suspected in view of the long interval between cases.

The first indication that cross contamination might have occurred came 9 months after the second case when REA demonstrated the identity of *C. diversus* isolates from the two cases. Environmental sampling at this time did not reveal any *C. diversus* contamination and routine testing revealed no further cases of *C. diversus* colonisation or infection amongst our neonates. The isolates from cases also differed by REA from the other 10 clinical isolates of *C. diversus* and the one *C. amalonaticus* strain, and the abscess-causing isolates (A-C) were biotype "d" whereas other isolates were generally of biotype "a" with one strain of biotype "e". Other workers have also found biotype

"d" to be predominant among abscess-causing strains.³ Brain abscess due to strains of biotype "a"³ and the rare biotype "e"¹⁰ have also been described, albeit less commonly. The 14 strains did not differ in antibiotic sensitivity pattern which was, therefore, of no value as a typing system, nor were SDS-PAGE of WCP and immunoblotting with NHS, both of which showed remarkable homogeneity among *C. diversus* strains. However, distinct banding patterns were obtained with the one *C. amalonaticus* strain by SDS-PAGE and this method may be useful for interspecies differentiation.

C. diversus may cause protracted outbreaks of neonatal meningitis and brain abscess involving a small number of cases over a long period of time.²¹ This may be due to its propensity for long term colonisation of the hospital environment or of hospital staff. REA is a useful epidemiological tool in the management of such outbreaks. Prolonged intravenous antibiotics combined with repeated CT scanning and drainage of the abscess cavities are used in the management of individual cases.

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