Witness Statement Ref. No. 224/3

NAME OF CHILD: CLAIRE ROBERTS

Name: Brian Herron

Title: Dr

Present position and institution:

Consultant Neuropathologist and Histopathologist,
Royal Victoria Hospital, Belfast Trust

Previous position and institution:
[As at the time of the child's death]

Senior Registrar, Neuropathology,
Royal Victoria Hospital

Membership of Advisory Panels and Committees:
[Identify by date and title all of those since your Witness Statement of 23rd December 2011]

None

Previous Statements, Depositions and Reports:
[Identify by date and title all of those since your Witness Statement of 23rd December 2011]

N/A

OFFICIAL USE:
List of previous statements, depositions and reports attached:

Ref:	Date:	
096-006-032	25.04.06	Deposition to the Coroner
224/1	19.12.11	Witness Statement to the Inquiry
224/2	23.12.11	Second Witness Statement to the Inquiry

IMPORTANT INSTRUCTIONS FOR ANSWERING:

Please attach additional sheets if more space is required. Please identify clearly any document to which you refer or rely upon for your answer. If the document has an Inquiry reference number, e.g. Ref: 049-001-001 which is 'Chart No.1 Old Notes', then please provide that number.

If the document does not have an Inquiry reference number, then please provide a copy of the document attached to your statement.

I. QUERIES ARISING OUT OF YOUR INITIAL INQUIRY WITNESS STATEMENT DATED 19TH DECEMBER 2011

With reference to your Inquiry Witness Statement dated 19th December 2011, please provide clarification and/or further information in respect of the following:

(1) Answer to Question 1 at p.2:

"I was appointed as a Registrar for Neuropathology in August 1991 and a Consultant in Neuropathology in September 1998.

Prior to 1998 I trained in general aspects of neuropathology in the Royal Victoria Hospital."

I would like to correct this date. I was appointed as a Registrar for Neuropathology in August 1992.

(a) State to the best of your ability the number of paediatric autopsies you had performed prior to 24th October 1996.

Between 1988 and 1992 I had performed paediatric autopsies. These were not specifically neuropathological cases. I have no record of the precise number but would estimate there were at least 100 over these 4 years. I had also presented the findings of many of these cases to neurological, paediatric, clinical and mortality meetings.

Between my appointment to neuropathology in August 1992 and Claire Robert's autopsy there were 36 paediatric neurology/neurosurgery autopsies in the Royal Victoria Hospital. I had full participation in 24 of these, and may have had some involvement in the remaining 12 cases at some stage during my training. I had published two papers and presented one paper relating to paediatric autopsy findings (references at end of deposition).

(b) Describe the examinations which you had taken in neuropathology prior to 24th October 1996.

I had passed national exams in general pathology in 1990, 1992 and 1993 for which I received the qualification DRCPath. Between 1992 and 1997 I was completing my training in neuropathology and sat my final exit exam in April 1997. Therefore, while I had trained in neuropathology for four years I had not completed this exam. In addition to the training in neuropathology I was undertaking a PhD in neurovirology within the Department of Neuropathology. I had published research papers and a book chapter on the topic of neurovirology. (References at end of deposition).

(c) **Describe all of your employment prior to 24th October 1996, giving the dates of employment, position and name of employer.**

Date	Grade	Employment Base
01/09/98 - Date	Consultant Histopathology & Neuropathology	Royal Victoria Hospital
01/03/96-31/08/98	Registrar/Senior Registrar, Neuropathology	Royal Victoria Hospital
01/08/94-28/02/96	Research Fellow, Department of Pathology	Royal Victoria Hospital
01/08/92-31/07/94	Registrar/Senior Registrar Neuropathology	Royal Victoria Hospital
01/12/92-31/07/92	Registrar, Histopathology	Belfast City Hospital
01/08/90-31/12/91	Senior House Officer, Histopathology	Belfast City Hospital
01/08/89-31/07/90	Registrar, Histopathology	Monklands District General Hospital, Glasgow
01/08/88-31/07/89	Senior House Officer, Laboratory Medicine	Walton/Fazakerley Hospital, Liverpool
01/08/87-31/07/88	Junior House Officer	Royal Victoria Hospital

(2) **Answer to Question 2 at p. 2:**

"I was a Senior Registrar during this period..."

(a) **Explain why your grade of Senior Registrar was not disclosed in:**

(i) **Claire's autopsy report.**

(ii) **The document entitled *"Provisional Anatomical Summary"* (Ref: 090-005-007).**

(b) **State whether a Senior Registrar's grade would normally have been disclosed in 1996 in:**

(i) **a paediatric autopsy report**

(ii) **a *"Provisional Anatomical Summary"***

(a&b)

I have gone through the records relating to autopsy reports from the Department of Pathology in the 1990s. The policy at that time was to enter the name but not the grade of the junior doctor on the autopsy report and provisional anatomical summary. The consultant's name was not recorded on these reports.

This policy has changed and the consultant's name is now recorded on all autopsy reports.

(3) **Answer to Question 3(a) at p.2:**

"I have no recollection of taking any photographs during Claire's autopsy, but photographs were taken during the brain cut. This would be routine procedure."

(a) **Please answer the question at (3) *"Please identify all notes, records... and images relating to Claire's autopsy that you made or were made on your instruction and provide a copy of them."***

I have provided a copy of the image from the brain cut to the Inquiry. I am aware that further histological (microscopic) images were taken by my colleague Dr Mirakhur. I understand these have been provided to the Inquiry.

(b) **Please clarify if it is *"routine procedure"* to take photographs during :**

(i) **anautopsy, and if so, state when and for what purpose.**

It would not be routine procedure to take photographs during an autopsy.

(ii) ***"the brain cut"***

It is routine procedure to take photographs during a brain cut.

(c) **Identify who took the photographs *"during the brain cut"* and state upon what date the photographs were taken.**

The photographs taken during the brain cut would have been taken by the biomedical scientist. I have no record of who this was. These would have been taken on 28/11/1996.

(4) **Answer to Question 5 at p. 2:**

"The date recorded for this autopsy was the 24/10/1996".

(a) **Explain what occurred on *"24/10/1996"* in relation to Claire's autopsy and what you did on that date.**

As this took place 16 years ago I have no specific memory of what happened in relation to Claire's autopsy on 24/10/1996.

(b) **State the dates of your involvement *"in the initial stages of the autopsy and the brain cut".* (Ref: WS 224/1 p.14 25(d)).**

The initial stages of the autopsy were on 24/10/1996 and the brain cut was on 28/11/1996.

(c) **State whether you were involved in drafting or preparing of the autopsy report on Claire, and if so, describe your involvement, the dates of that involvement and identify and produce any documents in which you were involved in preparing or drafting.**

As this occurred in 1996 I have no specific memories of the drafting or preparation of the autopsy report. I have signed the provisional anatomical summary and it is likely I was involved in its preparation.

(d) **In relation to your involvement *"in the initial stages of the autopsy and the brain cut"*. (Ref: WS 224/1 p.14 25(d)), state whether you took any steps to check or ascertain the accuracy and impartiality of the information in the autopsy request form, and in particular the information relating to Claire's clinical presentation, history, diagnosis and clinical problems on that form. If so, describe those steps, when you took them and what the outcome was. If not, explain why not.**

As this occurred in 1996, I have no recollection of any detail which can help to say whether I did or did not take any of the steps above.

(e) **State whether there was a *"review session at 1.45pm on the day of the autopsy"* as described on the autopsy request form and:**

(i) **If so, state when and where it was held, who was present and the outcome thereof, and produce any note, record or document relating thereto.**

(ii) **If not, explain why not.**

(iii) **State whether in 1996 there would usually have been a review session on the day of autopsy, and if so, state the purpose thereof, who would usually attend and what record of that session would normally be made.**

(i – iii)

There had previously been a tradition in the Department of Pathology to hold review sessions relating to autopsies. This had not been the case for many years and I do not believe that these review sessions took place in 1996. More specifically, it is highly unlikely, that even if the review sessions did exist at that time that Claire's case would have been subject to a review. This was a brain only autopsy and because of the fragility of the brain further handling at this stage would have damaged the tissue.

(5) **Answer to Question 6(a) at p. 3:**

"I have no recollection whether I did or did not speak to any of the above named...."

(a) **State whether it was or would likely have been your practice in 1996 and prior to 11 February 1997 to have any communications in relation to an autopsy or autopsy report with the clinicians who had care of the paediatric patient, and if so, state with whom, when and how this would normally occur, the purpose of these communications, and any record or note normally made of those communications.**

The neuropathologists have always been active at disseminating information relating to biopsies in living patients and also autopsies in deceased patients. Although we comprise

about 3% of the clinical neuroscience staff, we present about 25% of the clinical cases at neuroscience meetings. This was the tradition before 1996 and it remains the case.

There would have frequently been dialogue between the neuropathologists and the clinical team who cared for the patient. With specific regard to neuropathology cases, it has always been the practice to communicate the pathology report with the clinicians by means of clinical presentation. The two main channels of communication in 1996 were the Paediatric Mortality Meetings and the Neuroscience Grand Rounds (NSU). As far as I can remember, all paediatric deaths in the hospital were presented at the Paediatric Mortality Meetings. I am not entirely certain that these meetings had begun in 1996, but it is my impression they had. In addition, neuropathology autopsy reports were regularly presented at the NSU. This is a weekly meeting attended by neurologists, paediatric neurologists, neurosurgeons, neuroradiologists and neuropathologists. The paper work suggests that Claire's case was to be discussed at one of these NSU meetings. I have no record of who did this presentation and whether or not I was in attendance. No attendance record was taken at these meetings.

These presentations are usually done after a final report is completed or sometimes to gather clinical information to allow the final report to be completed.

Answer to Question 7(a) at p. 3:

"As I was not the author of the final report..."

Some general information regarding the performance and drafting of autopsy reports is needed in order to understand the answers to the remaining questions. What follows is a description of the process of performing an autopsy and the relationships between the doctors involved.

If a request for an autopsy arrives in the Department, either the junior doctor or consultant is informed, usually by the mortuary staff. The junior doctor and consultant discuss the case and what procedures should follow. The junior doctor, under the supervision of the consultant, may perform many of the technical aspects of the autopsy. These technical aspects may include examination of the body and removal of the organs. The consultant is fully involved or informed of the outcome of these procedures.

The provisional anatomical summary of the initial findings is prepared either by the junior doctor for the consultant to check, or by the consultant alone.

After a period of time, if the brain has been retained, a second examination is made and attended by the junior doctor and consultant. A further examination of the brain takes place and small pieces of tissue called tissue blocks are taken from the brain. These are processed in the laboratory and made into histological slides of tissue. When these histological slides are available, the junior doctor will look at them and take them to the consultant to discuss. This is called "signing out". Either the junior doctor in consultation with the consultant or the consultant alone will then write a final report. The consultant therefore is involved at all stages of the autopsy, has seen all reports generated with regard to the autopsy and will have approved these before they are despatched to the clinical staff. The notion that one doctor starts the case and another finishes it, would be incorrect for the reasons above.

(b) **Explain why you are the sole named pathologist on Claire's autopsy report if you are *"not the author of the final report".***

As explained earlier it was the policy of the Department to record the junior doctor's name on an autopsy report. This policy has changed. It is now the consultant pathologist who is named on any report as they take ultimate responsibility for the report.

(c) **Explain why you gave sworn oral evidence at Claire's Inquest as the author of Claire's autopsy report if you are *"not the author of the final report"*.**

Although I did assume I was the author of the final report I am not aware that I gave sworn oral evidence at Claire's inquest as the author of Claire's final autopsy report. I certainly gave evidence as I was involved in Claire's autopsy and had reviewed the case before the Inquest. I often gave evidence at Inquests as a senior registrar in cases I had been involved in, supervised by consultants. There is a list of doctors who can do this in Northern Ireland. The current list could be obtained from the Coroner's office.

State at what date between 24th October 1996 and 12th February 1997 your involvement in Claire's autopsy and autopsy report ceased.

As this case was in 1996 I have no recollection on what date my involvement ceased or if indeed I did cease to be involved in this case.

(d) **Identify the parts,processes and examinations of/during the autopsy in which you were not involved in Claire's case.**

(e) **Describe your input to or involvement in an autopsy report in Claire's case, whether final or draft, and when this occurred.**

(d, e &f)

As this case was in 1996 I have no recollection of the specific details of my input to the reports in this case. I was involved in the initial autopsy and the brain cut.

(f) **Describe all other involvement you had between 11th February 1997 and 4th May 2006 in Claire's case, including her autopsy and autopsy report.**

There is correspondence from various sources that relate to Claire's autopsy (copies provided). The correspondence appears to begin on 16/12/2004. This correspondence relates to the referral of Claire's case to the Coroner and events relating to the subsequent inquest in 2006. In preparation for the inquest I reviewed the pathological findings in the case and the expert opinions of other doctors. These expert opinions are those seen on the Inquiry website.

(g) **State whether after your involvement *"in the initial stages of the autopsy and the brain cut"* (Ref: WS 224/1 p.14 25(d)) you were shown or saw Claire's autopsy report, or any draft thereof, prior to 12th February 1997, and if so, when, in what circumstances did you see or were shown the report, by whom and what, if anything, you did as a result of seeing it. If not, explain why you did not see or were not shown it.**

As this case was in 1996 I have no recollection of my involvement.

(h) **Explain why the final report is not signed.**

The final report would not have been sent had it not been signed. The signed report is the copy that goes to the clinician and to my knowledge a final report has never left

neuropathology unsigned. The reports that are attached to the PDF document with these questions are likely to be either draft reports or reports subsequently printed directly from computer screen.

(i) **Explain how and why you were involved *"in the initial stages of the autopsy and the brain cut"* (Ref: WS 224/1 p.14 25(d)) but not thereafter, and state whether this was usual practice in paediatric brain only autopsies, and state the reasons for your answer.**

(j) **Identify who determined that you would only be involved *"in the initial stages of the autopsy and the brain cut"* (Ref: WS 224/1 p.14 25(d)) and that another clinician would have conduct of the autopsy thereafter, and state when this decision was made and where it is recorded. Please furnish a copy of that record.**

(k) **Explain how responsibility for Claire's autopsy was transferred from you to another clinician and where this is recorded. Please furnish a copy of that record.**

(i,j and k) This answer refers to these three points

Some general information regarding the performance and drafting of autopsy reports is needed in order to understand the answers to the remaining questions. What follows is a description of the process of performing an autopsy and the relationships between the doctors involved.

If a request for an autopsy arrives in the Department, either the junior doctor or consultant is informed, usually by the mortuary staff. The junior doctor and consultant discuss the case and what procedures should follow. The junior doctor, under the supervision of the consultant, may perform many of the technical aspects of the autopsy. These technical aspects may include examination of the body and removal of the organs. The consultant is fully involved or informed of these procedures.

The provisional anatomical summary of the initial findings is prepared either by the junior doctor for the consultant to check, or by the consultant alone.

After a period of time, if the brain has been retained, a second examination is made and attended by the junior doctor and consultant. A further examination of the brain takes place and small pieces of tissue called tissue blocks are taken from the brain. These are processed in the laboratory and made into histological slides of tissue. When these histological slides are available, the junior doctor will look at them and take them to the consultant to discuss. This is called "signing out". Either the junior doctor in consultation with the consultant or the consultant alone will then write a final report. The consultant therefore is involved at all stages of the autopsy, has seen all reports generated with regard to the autopsy and will have approved these before they are despatched to the clinical staff. The notion that one doctor starts the case and another finishes it, would be incorrect for the reasons above.

(6) **Answer to Question 8(c) at p.4:**

"I did not create the histology slides, these would have been created by the Biomedical Scientists(previously called Medical Laboratory Scientific Officers (MLSOs). There is an indication they were prepared on 23/01/1997."

(a) **Identify the *"Biomedical Scientists"* who created the histology slides.**

It was not the policy in 1996/97 that the biomedical scientist who created the slides was individually identified. It is now policy that the individual biomedical scientists who create a slide are identified by recording their initials on the slide.

(b) **State where it is identified that *"they were prepared on 23/01/1997"* and provide a copy of that indication.**

See Page 090-054-189 column "Date Out" - This refers to the date that the slides would have gone to the pathologist.

(7) **Answer to Question 8(d) at p.4:**

"There are many complicated procedures necessary in the preparation of a Neuropathology report. It took until 11/02/1997 for these to be completed."

(a) **Explain the *"complicated procedures"* that were *"necessary in the preparation of a Neuropathology report"*, and the dates when each of these procedures took place in Claire's case.**

Some general information regarding the performance and drafting of autopsy reports is needed in order to understand the answers to the remaining questions. What follows is a description of the process of performing an autopsy and the relationships between the doctors involved.

If a request for an autopsy arrives in the Department, either the junior doctor or consultant is informed, usually by the mortuary staff (24/10/1996). The junior doctor and consultant discuss the case and what procedures should follow. The junior doctor, under the supervision of the consultant, may perform many of the technical aspects of the autopsy. These technical aspects may include examination of the body and removal of the organs. The consultant is fully involved or informed of these procedures.

The provisional anatomical summary of the initial findings is prepared either by the junior doctor for the consultant to check, or by the consultant alone.

After a period of time, if the brain has been retained, a second examination is made and attended by the junior doctor and consultant (28/11/1996). A further examination of the brain takes place and small pieces of tissue called tissue blocks are taken from the brain. These are processed in the laboratory and made into histological slides of tissue. When these histological slides are available, the junior doctor will look at them and take them to the consultant to discuss. This is called "signing out". Either the junior doctor in consultation with the consultant or the consultant alone will then write a final report.

Answer to Question 9(a) at p. 4:

"For clarification the Neuropathologist understands the Terms "Anatomical Summary" and "Clinical Summary" to have different meanings. The term Anatomical Summary is used by the Neuropathologist to summarise the post mortem examination findings after its completion ..."

(b) **State whether you are the author of the document entitled *"Provisional Anatomical Summary"* (Ref: 090-005-007).**

It is likely that I was the author of the Provisional Anatomical Summary as I have signed it.

(c) **Identify the signature on that document. (Ref: 090-005-007).**

This is my signature.

(d) **State the date of the document entitled *"Provisional Anatomical Summary"* (Ref: 090-005-007).**

As it appears photocopied on the website it appears undated. However I do not have the original report to check if it is actually dated or undated.

State whether that document is complete and state the reasons for your answer. If it is not complete, please furnish a copy of the complete document.

It appears complete although I do not have the original document to verify this.

(e) **Identify the source of the information on that document that Claire's *"Date of Admission"* was *"22/10/96"*.**

This may have come from a number of sources including the Royal Victoria Hospital autopsy request form, telephone conversation, hospital notes etc. As this was in 1996, I cannot recall from where this information was taken or by whom.

(f) **Identify the source of the information on that document that the *"Time of Death"* was *"6.25hrs"*, and explain whether that refers to 06.25hrs or 18.25hrs.**

It refers to 18.25hrs. There are a number of possible sources for this information. The autopsy request form has the time 18.15hrs recorded. This is in small handwriting and could be misinterpreted as 18.25hrs (this is more apparent on the original document than the photocopies). It may have been taken from other sources such as a telephone call or from the hospital notes. It is unclear who was responsible for obtaining this information This answer expands on Q7(b) from deposition 224/1

(g) **Identify the source of the information on that document that the clinicians responsible for Claire were *"Dr.Webb/Dr. Steen"*.**

The autopsy request form was signed by Dr Heather J Steen. Other than that, I have no recollection as to the source of this information in 1996 or who initially recorded this informtion.

(h) **Identify the source of the information that Claire had a *"History of acute encephalopathy"*.**

History of acute "encephalopathy" is an interpretation of the clinical information described under "history of present illness" in the autopsy request form.

(i) **State whether Claire's medical notes, records, CT scan and chest X-rays were available to be examined either at the time this document was produced or thereafter. If you cannot recall, please state whether they were likely/normally available and if so, for what purpose.**

As this case was in 1996 I cannot recall if they were available in part or in total, before or after the autopsy. In some they are available and in others they are not readily available.

(8) **Answer to Question 10(b) at p.5:**

"I have no recollection whether or not the medical notes were available in part or in totality before the post mortem".

(a) **Explain what medical notes and records would normally have been available to you in 1996 and 1997:**

(i) **before you performed a post-mortem examination**

In some cases all of the medical notes were available. In other cases only an autopsy request form was available. In some cases the history is obtained by telephone or face to face conversation.

(ii) **After you performed and before you provided an autopsy report.**

The medical notes and records would often have been available between performing the autopsy and providing a report. They may not have the results of all laboratory or radiological investigations attached however, particularly if performed shortly before death.

(9) **Answer to Question 11(b) at p. 5:**

"There is no recording of the brain weight before fixation. Paediatric brains are extremely fragile at the time of autopsy and easily damaged by handling. There are often transferred directly into fixative without any further analysis in order to protect their structure."

(a) **State whether it was usual or normal practice in 1996 and 1997 to measure the fresh brain weight in:**

(i) **A paediatric brain only autopsy**

(ii) **A paediatric autopsy where cerebral oedema has been diagnosed as the cause of death.**

(b) **State in what circumstances would it have been appropriate to have weighed the "fresh" brain weight in a paediatric brain only autopsy in 1996.**

(c) **Explain why you did not measure Claire's fresh brain weight.**

(d) **Describe whether and how measuring Claire's fresh brain weight may have assisted in the autopsy and state the reasons for your answer.**

(a - d)

It was always Departmental policy to weigh the brain at time of cut up. Depending on the 'state' of the brain at the time of autopsy it is sometimes possible to weigh it then. However, and in particular, when there is cerebral oedema there are a number of recognised procedures for removing the brain that do not allow for accurate weighing at that stage. An oedematous baby's brain has the consistency of "boiled fish" and is therefore extremely fragile. Any sort of handling at the time of autopsy could damage the tissue and create artefact that may interfere with the interpretation of the pathological findings.

Brain weight is useful to record but is not *per se* an accurate indicator of pathological processes or oedema. There is a large variation in brain weights for the normal population. The statistics against which brain weights were compared are a guide but were not

necessarily relevant to the population of Northern Ireland or a population of children born when Claire was born.

There are diseases which can increase or decrease brain weight in the absence of cerebral oedema.

Cerebral oedema is better diagnosed by examining a carefully fixed brain weeks after the initial autopsy, by looking for swelling of the surface of the brain (gyri, unci and tonsils) and by microscopic examination of the brain.

(10) **Answer to Question 14 (a) at p. 6:**

"The consent was signed by Claire's father with an indication that Dr. Heather Steen was the Consultant."

(a) **Explain what you mean by *"the Consultant"*.**

A consultant in the Health Service is a permanent, senior member of medical staff.

(b) **State the significance of the *"indication that Dr. Heather Steen was"* named as *"the Consultant"*on *"[t]he consent"* and the responsibilities of that named consultant.**

A consultant can be a physician or surgeon, radiologist, pathologist etc. This includes a paediatric physician or paediatric surgeon. The space in the consent form entitled "physician" or "surgeon" had Dr Heather Steen's name in it. I am not party to a specific job description for a consultant in the Health Service in 1996.

(11) **Answer to Question 14(b) at p. 7:**

"The decision to limit the autopsy to brain only was the responsibility of the relevant clinicians."

(a) **Identify *"the relevant clinicians"* in Claire's case and state the reasons for your answer.**

The consent form records Dr Steen's name in this case. There may have been other doctors involved in the consent taking process whose names are not recorded and whose participation in the consent I have no knowledge or record of. This does not mean there others were not involved.

The consent is taken in consultation with the family and the consent form clearly indicates, and it is underlined, that the autopsy should be limited to brain only.

(12) **Answer to 14(e) at p. 7:**

"In as far as I was involved with this autopsy there would be no specific reason to have extended this to a full autopsy. The autopsy was done to address the presence or absence of status epilepticus and encephalitis. The limited consent for the autopsy was adequate to address these issues."

(a) **State the reasons to extend a paediatric brain only autopsy to a full autopsy in 1996.**

A full autopsy could only have been performed if there was appropriate consent to do so. It could not have been extended if there was consent for a brain only autopsy. If appropriate

consent to extend the autopsy had been obtained to address a specific issue, this may have been a reason to do so.

(b) **Explain all of the matters/issues which *"[t]he autopsy was done to address"* and state the reasons for your answer.**

(i) In particular please clarify whether you are stating that Claire's autopsy was performed solely *"to addressthe presence or absence of status epilepticus and encephalitis"* andstate the reasons for your answer.

(ii) If so, identify the person/s who determined that this was the sole purpose of Claire's autopsy, and state when this was determined, identify any record or note of the sole purpose of the autopsy and state whether Claire's parents were informed that this was the sole purpose of the autopsy, and if so, state when they were told and by whom.

(iii) If not, describe the other purposes of Claire's autopsy and state the reasons for your answer.

(i-iii)

The neuropathologist is not involved in the consent taking process and I am therefore unable to answer many of the questions above. A neuropathologist is guided by the autopsy request form. Page 3 of the Royal Victoria Hospital autopsy request form indicates four clinical problems; cerebral oedema, status epilepticus, inappropriate ADH secretion and viral encephalitis. On page 2 the clinical diagnosis was cerebral oedema secondary to status epilepticus. These were the specific issues highlighted on the autopsy request form and the autopsy would have been performed to address these issues. If any further abnormality had been detected it would have been recorded.

(c) **State whether a full autopsy examination may have been relevant or assisted in:**

- **increasing medical knowledge in Claire's case or generally**
- **ascertaining the cause of death and/or in and in particular in relation to :**

(i) cerebral oedema

(ii) status epilepticus

(iii) encephalitis, viral or otherwise

(iv) Inappropriate ADH secretion

(v) Hyponatraemia

And state the reasons for your answer.

(i-v)

The autopsy, as performed, addressed the issues of cerebral oedema, status epilepticus and encephalitis.

Inappropriate ADH secretion is a condition that is better diagnosed in life and not at autopsy.

Hyponatraemia is a condition better diagnosed in life and not at autopsy.

I have no reason to believe that extending the autopsy would have increased medical knowledge or further assisted in ascertaining the cause of death.

(13) Answer to Part II at p.7:

"When the Coroner's deposition was taken it was assumed that I had actually been the author of the final report [...]. When retrieving documentation subsequent to this it has become apparent that I was not the author of this final report. The deposition therefore was made under these circumstances. See 25d."

*"...With specific regard to Claire's autopsy, I was involved in the initial stages of the autopsy and the brain cut. Until I retrieved the documents that allowed me to prepare this report, I assumed I had also written the final report referred to in this deposition. It now seems this is not the case and it was written by one of the Consultants in the Department at the time."***(Ref: WS-224/1 p. 14, 25(d)).**

(a) Explain the dates or period to which you refer when you state *"whenretrieving documentation subsequent to this"*.

(i) In particular, please clarify whether you refer to the period following your receipt of the Inquiry's first set of witness questions to you when you say *"[u]ntil I retrieved the documents that allowed me to prepare this report..."*

(ii) State the date when you first received the Inquiry's first set of witness questions to you (Ref: WS-224/1).

(i-ii)

I was asked to prepare a statement (WS 224/1) for the Inquiry on 01/12/2011. It was in the preparation of this statement that I realised that I had not been the author of the final autopsy report. In particular it became apparent when papers were retrieved from off-site storage that there were draft reports edited by my colleague Dr Mirakhur.

(b) State what *"documentation"* you retrieved.

(c) State whether you retrieved this *"documentation"* prior toyour deposition to the Coroner on 25th April 2006 and state the reasons why/not.

(d) State when you first saw a copy of the final autopsy report (Ref: 090-003-003).

(e) State when you were first aware that you were the sole named pathologist in that report.

(i) At that time, state whether you were aware that you were *"not the author of the final report"*, and explain the reasons why.

(f) State when you first became aware that you were *"not the author of the final report"*.

(b-f)

In preparation of my deposition 224/1, documents were retrieved from off-site storage. These documents included draft reports of the final autopsy report. I had not previously been aware of the existence of these draft reports. I was not aware of these prior to my deposition to the Coroner on 25th April 2006.

I had seen a copy of the final autopsy report before 25th April 2006. I assumed I was the author of the final report up until 2011 and I only became aware that I wasn't in 2011.

(g) **Explain why you did not inform:**

(i) The Coroner

(ii) The Inquiry into Hyponatraemia-Related Deaths

that you were *"not the author of the final report"* prior to your Witness Statement to the Inquiry dated 19th December 2011.

I only became aware that I was not the author of the final report in preparation of my deposition in 2011.

(h) **Describe what preparation you made to re-acquaint yourself with Claire's autopsy before your sworn deposition to the Coroner on 25th April 2006 at Claire's Inquest.**

(i) State what documentation, notes and records you read or examined prior to 25th April 2006 to familiarise yourself with Claire's autopsy and produce copies of those documents.

(ii) In particular state whether you examined the notes and records that have been produced by the Regional Neuropathology Service in relation to Claire's autopsy (Attached hereto). If so, when did you examine them. If not, state the reasons why not.

(iii) State the basis upon which you assumed you *"had actually been the author of the final report"*.

(iv) State whether in 2006 you would normally have checked your notes and records relating to a paediatric brain only autopsy before making a sworn deposition to the Coroner at an Inquest and state the reasons why.

(v) State whether in 2006 at the time of and during Claire's inquest you had any recollection of carrying out Claire's autopsy and writing the autopsy report, and if so, describe that recollection.

(i-v)

Prior to any Inquest I would review any available paperwork or notes relating to an autopsy. I would often also review the histological slides. I do not remember specifically what was done before the Inquest in 2006, but I do recall reviewing the case in detail. There were further reports by other experts that were necessary to read. I remember reviewing these reports. I also read the final autopsy report and reviewed the slides. I cannot recall that in 2006 I did or did not recall carrying out Claire's autopsy in 1996. As my name was the only name on the report I assumed I

was the author. Also, following review of the case I agreed with the commentary and conclusion in the case (as written in the final autopsy report).

If you were involved only *"in the initial stages of the autopsy and the brain cut"* explain:

- **your actual knowledge of each of the following statements**
- **the basis upon which you gave sworn evidence at Claire's Inquest that:**

As the case was in 1996 I do not recall if I was involved only in the initial stages of the autopsy and the brain cut for the reasons already discussed. I had also reviewed the case in detail before the Inquest.

(vi) *"I found cerebral oedema..."* **(Ref: 091-005-019)**

The presence of cerebral oedema would have been noted at the time of the autopsy and the brain cut and I would have been aware of this at these stages of the autopsy. I had also reviewed the case in detail before the Inquest.

(vii) *"There was mild inflammation of the brain. I did not find any virus to cause this though that does not exclude a virus."***(Ref: 091-005-019)**

I had reviewed the case before the Inquest and found mild inflammation of the brain and that no virus was found.

(viii) *"I was not thinking of fluid management but SIADH."* **(Ref: 091-005-019)**

In preparation for the Inquest I was aware that SIADH was an issue.

(ix) *"The main pathology finding was cerebral oedema with a little inflammation in the brain. In a typical case of encephalitis the degree of inflammation is more severe."* **(Ref: 091-005-019)**

I had reviewed the case and was able to make these comments based on my review.

(x) *"I weighed the brain. It was heavier than normal but there had been abnormal development of the brain.1300grams would have been expected - Claire's was 1606"* **(Ref: 091-005-019)**

I had weighed the brain at the brain cut and was able to make this comment based on that observation.

(xi) *"In addition there was some brain inflammation - possibly a viral infection."* **(Ref: 091-005-019).**

(xii) *"It could have resulted from a gastro-intestinal infection."* **(Ref: 096-006-034)**

(xiii) *" I would have expected an infection to be the underlying cause."* **(Ref: 096-006-034)**

(xiv) *"A metabolic cause could not be excluded."* **(Ref: 096-006-034)**

(xv) *"It is difficult to say what part, if any, her epilepsy played in her death."***(Ref: 096-006-034)**

(vi – x)

I had reviewed the case before the Inquest and was able to make these comments based on this review.

(14) **Answer 15(a),(b),(d), (e) and (g) at p. 8:**

"(a,b and d) As a Neuropathologist it is more appropriate for me to describe pathological findings and leave it for the expert clinicians to come to overall conclusions in this specific case...

(c and e) As this was not my final report I cannot comment on these questions

(g) As I was not the author of the final report I defer answering the question."

(a) **The questions at 15(a),(b),(d), (e) and (g) arise from your sworn deposition at Claire's inquest. Please provide complete answers to these questions. If you are unable to do so, explain the reasons why.**

I do not have verbatim records of the Inquest and it is not possible to know from the complete discussion these comments were taken from. My deposition as recorded from the Inquest is clearly fragments taken from the evidence. The context of the recorded comments and in particular the questions that led to the comments are not known.

With regard to a, b, and d I do not recall giving a definitive cause of the cerebral oedema at the Inquest

(b) **Questions 15(e) and (g) arise from your oral deposition to the Coroner at Claire's inquest. Please answer the question fully. If you are unable to do so, explain the reasons why.**

(e and g). I had reviewed the case and had not made a conclusion as to the most likely cause of the inflammation or the significance of the degree of inflammation

(15) **Answer to Question 15(h)(i) at p.9:**

"These have been identified in the report by Dr. Evans

CNS Disorders...

Malignancy: Lung, brain, pancreas...ovary. lymphoma, leukemia, thymoma

Endocrine Disorders:..

Drugs...."

(a) **You have not answered Question 15(h) which arises from your sworn oral deposition to the Coroner. Explain why you were *"not thinking of fluid management but SIADH"*, and explain what difference it would have made if you considered *"fluid management"* at the time of your involvement in Claire's autopsy and/or report.**

I do not have a verbatim transcript of the Coroner's Inquest and the testimony as recorded is most likely a short summary of statements that were made at the Inquest and the context of these questions is not recorded. There was a clinical suggestion of SIADH and that would have prompted me to consider it.

It is more appropriate to ask experts in fluid management the second part of the above question as it relates to issues better made by investigations in life rather that at autopsy.

(b) **If the causes of SIADH manifest in other parts of the body and *"Inappropriate ADH secretion"* was listed in the Autopsy request form as a clinical problem and part of the clinical history in Claire's case, please explain why a limited post-mortem was appropriate in Claire's case.**

The autopsy addressed the issues indicated on the autopsy request form. There were causes of SIADH within these indications. In addition the diagnosis of SIADH is not one that can be made at autopsy.

(16) **Answer to Question 15(h)(ii) and (iii) at p. 10:**

"(ii) I have no recollection if I did or did not know of the findings of a chest x-ray at the time of autopsy...

(iii) I have no recollection whether I did or did not know the chest x-ray findings. The autopsy was performed to investigate the presence of status epilepticus and underlying encephalitis and was therefore adequate to address those questions."

(a) **State whether chest x-ray findings would usually/normally have been available in 1996 at the time of a paediatric autopsy.**

(b) **State whether you would have expected Claire's chest x-ray findings to have been available to you when you were involved in her autopsy.**

(a& b)

In some cases they may have been available and in others they may not have been available.

In the copy of the chest x-ray report on the Hyponatraemia website, only one of 2 pages is present. There is no hand-signed indication of when it was received by the clinicians. I have checked this point in Claire's notes. There is only one of 2 pages and it is not signed or dated.

There is no indication it was in the notes when the autopsy was performed on when the final report was sent out.

In 1996 there was not the integrated computer system in the hospital available today. In general a child may have had an xray performed. It may or may not have been subject to a verbal report. It would then have a formal report. This would be sent for typing. Once authorised by the radiology consultant it would have been put into internal mail or hand delivered. This can take several days. Results done in life were commonly not in the hospital notes by the time of autopsy or even by the time of report completion. There was no integrated computer system to know a test result of even if a test had been done.

Also as mentioned above Claire's respiratory examination was normal after she became hyponatraemic suggesting that if any respiratory disease was subsequently present it was not the cause of the Hyponatraemia.

(c) **Explain the basis for your statement that *"The autopsy was performed to investigate the presence of status epilepticus and underlying encephalitis".***

These were the indications on the autopsy request form.

(d) **Explain the purpose/s of Claire autopsy and state the reasons for your answer.**

(i) **In particular, explain whether the purpose of Claire's autopsy's was solely *"toinvestigate the presence of status epilepticus and underlying encephalitis"*and state the reasons for your answer.**

(ii) **Identifythe person/s who determined the purpose/s of Claire's autopsy and state the reasons for your answer.**

(iii) **Identify the record or note of the purpose of Claire's autopsy and furnish a copy of it.**

(i-iii)

The neuropathologist is guided by the autopsy request form. This has been made available to the Inquiry. The autopsy was performed appropriately to address the diagnoses on the autopsy request form. If any other pathological finding had been discovered it would have been recorded. The Neuropathologist is not involved in the consent taking.

(e) **Identify the matters which Claire's autopsy was investigating other than *"the presence of status epilepticus and underlying encephalitis"*and state the reasons for your answer.**

The neuropathologist is guided by the autopsy request form and the diagnoses relate to cerebral oedema, status epilepticus, inappropriate ADH secretion and viral encephalitis (Page 3 of autopsy request form). If any other disease had been found it would have been recorded

Answer to Question 17 (a)-(c) at p. 11:

"(i and ii) Before commencing an autopsy the Pathologist usually has a consent form and a clinical summary. Often, but not always, some or all of the clinical notes are available...

(b and c) When there is a death, particularly the death of a child, it is important that the autopsy is performed in a reasonable time. This not only facilitates the burial and funeral arrangements, but also prevents decomposition of the body. The longer the period of time between the death and the autopsy, the greater the degree of decomposition and the less reliable will be the pathological results. The Pathologist is guided by the clinicians as to the important aspects of any particular case. While it may be considered preferable to have all of the notes and time to read all of the notes in every case, this is neither possible nor practical. ... Many charts, particularly in someone with a long history of illness may run to several volumes and hundreds if not thousands of pages of detailed medical information and test results. If the Pathologist was expected to read and dissect these in detail it could be several weeks before any autopsy was performed."

(f) **State whether you would have expected Claire's medical notes and nursing notes to have been available prior to or at the time of Claire's autopsy and state the reasons for your answer.**

These are often made available before the autopsy or sometimes afterwards. As this case was in 1996, I do not recall if in this particular instance they were made available before or afterwards.

(g) **State whether Claire's clinical notes were read, or if you do not recall, whether they were likely read prior to or at the time of her autopsy and state the reasons for your answer.**

As this case was in 1996, I have no recollection.

(h) **Explain the relevance, if any, of your statement that *"When there is a death, particularly the death of a child, it is important that the autopsy is performed in a reasonable time. This not only facilitates the burial and funeral arrangements, but also prevents decomposition of the body. The longer the period of time between the death and the autopsy, the greater the degree of decomposition and the less reliable will be the pathological results"* to Claire's case where there was a brain only autopsy.**

This statement is self-explanatory. I am unclear what further information is being requested. This information is as relevant to a brain only autopsy as to a full autopsy.

(i) **Explain whether, and if so, how, you were *"guided by the clinicians as to the important aspects of"* Claire's case.**

Yes. From the information on the autopsy request form.

(j) **Explain what, if anything, you did, or if you cannot recall, what you likely/normally would have done, in 1996 to ensure that any guidance from the clinicians *"as to the important aspects of"* Claire's or any other case was accurate and impartial, particularly where there may have been an issue over the conduct of the clinicians and their involvement in the child's death.**

As this case was in 1996, I have no recollection.

(17) **Answer to Question 19 at p.12:**

"Although I did not write the final autopsy report, I was involved in the original autopsy and took samples of CSF which I understand did not show any specific infection."

(a) **Explain what you mean by *"the original autopsy"* and state the date when it occurred.**

This refers to the initial stages of the autopsy (24/10/1996). Samples for virology are taken at the time of the autopsy in the mortuary.

(b) **Describe your involvement in taking *"samples of CSF"*and in particular whether you took the CSF samples.**

As this case was in 1996, I do not recall this specifically, but it is likely that I took the samples.

(c) **State the date when CSF samples were taken in Claire's autopsy, and if you do not recall, then state the date when they were likely/normally taken and/or the stage at which they was taken during the autopsy.**

24/10/1996. As this case was in 1996, I cannot recall specifically but the samples are usually taken just before the brain is removed.

(d) **Identify any others involved in taking these samples.**

See 17 (B) above.

(18) **Answer to Question 25(a) at p. 14:**

"I am happy to provide copies of documents held regarding the case of Claire Roberts in the department."

(a) **Please furnish copies of all documents relating to Claire Roberts.**

There have been furnished. I attach further correspondence relating to the referral of Claire's case to the Coroner.

(19) **Answer to Question 25(d) at p.14:**

"At the time of Claire's death and autopsy I was a Senior Registrar in Neuropathology. My work was supervised by a Consultant Neuropathologist. With specific regard to Claire's autopsy, I was involved in the initial stages of the autopsy and the brain cut. Until I retrieved the documents that allowed me to prepare this report, I assumed I had also written the final report referred to in this deposition. It now seems this is not the case and it was written by one of the Consultants in the Department at the time."

(a) **Identify the Consultant Neuropathologist who supervised your work in between October 1996 and your appointment as a Consultant in September 1998.**

Dr M Mirakhur.

(b) **Explain the nature and degree of supervision of your work during that period.**

All work in neuropathology is consultant led and supervised.

(c) **Describe the involvement, if any, of that supervising Consultant Neuropathologist in your work in relation to Claire's autopsy and autopsy report.**

As this case was in 1996, I do not recall any specific detail with regard to supervision in Claire's case. I have made general points relating to the interaction between junior doctor and consultant in 1996.

(d) **Explain what are *"the initial stages of the autopsy"*, what you did at the initial stages of Claire's autopsy and when you did this.**

This refers to the procedures in the mortuary on 24/10/1996. As this was in 1996, I have no recollection of the specifics of my involvement, but routinely I would have been there at the autopsy and performed most of the technical procedures relating to it.

(e) **Explain what is involved in "the brain cut", what you did and when you did this.**

The brain cut is a further stage of examination of the brain. After the autopsy the brain is fixed in formalin in order to for it to harden. Subsequently a separate examination of this brain is performed and this was done on 28/11/1996. During the brain cut the case is photographed and small blocks of tissue are taken for further laboratory processing.

(f) **Identify the Consultant who you believe wrote *"the final report"*,explain the basis for your belief and why that report is not signed by that Consultant.**

Dr M Mirakhur, based on the editing of the draft reports. As stated above, to my knowledge, a report has never left the department unsigned.

II. QUERIES ARISING OUT OF YOUR SECOND INQUIRY WITNESS STATEMENT DATED 23RD DECEMBER 2011

With reference to your second Inquiry Witness Statement dated 23rd December 2011, please provide clarification and/or further information in respect of the following:

(20) **Answer to Part I at p.2:**

"When the paperwork was retrieved to prepare these depositions, it was discovered that one of my colleagues (Dr M Mirakhur) was the author of the final report. There will therefore be parts of the autopsy report on which I can comment and others that are more appropriately addressed by Dr Mirakhur."

(a) **Specify *"the paperwork"* which *"was retrieved to prepare these depositions"* and provide copies thereof.**

These are the draft copies of the final report. Copies have been provided.

(b) **State when, by whom and from whom/where this *"paperwork was retrieved"*.**

By the archivist, Miss C Kilpatrick from off-site storage. These files were requested by me on 6/9/2011. I am not sure when they were received. This was prompted by a letter sent to the NIRFM regarding cases for the Inquiry. The Inquiry reference is BC=0051-11

(c) **State whether this paperwork was either available to or examined by you prior to your deposition at Claire's inquest and state the reasons for your answer.**

I did not have sight or knowledge of the draft reports before the Inquest.

(d) **Identify who *"discovered that one of [your] colleagues (Dr M Mirakhur) was the author of the final report"* and the date when this was discovered.**

I discovered this while preparing deposition S 224- 1.

(e) **Describe the respective involvement in Claire's autopsy of:**

(i) **Yourself**

(ii) **Dr.Meenakshi Mirakhur**

including what procedures / examinations / tests you each carried out.

I have previously described the typical involvement of a junior doctor/consultant in performing an autopsy. There is evidence I was involved in the autopsy on 24/10/1996 and in the brain cut on 28/11/1996. I may have been involved in preparing further documents or in discussions, but I do not remember this specifically.

(f) **State specifically which findings in the autopsy report are:**

(i) **Your findings**

(ii) **The findings of Dr.Meenakshi Mirakhur**

(i& ii)

The autopsy report and procedures are supervised by the consultant. I was involved in the initial autopsy and the preparation of the provisional anatomical summary and the brain cut. As the case was in 1996 I cannot recall my involvement in other aspects of the case.

(g) **Describe any discussions you had regarding Claire's autopsy with Dr.Meenaskhi Mirakhur prior to completing the autopsy report. If so, identify who else was present and state when they took place, their content and where they are recorded.**

As the case was in 1996, I have no recollection of discussions at this time.

(h) **Explain why you, rather than Dr.Meenakshi Mirakhur:**

(i) **Provided a Deposition to the Coroner**

(ii) **Delivered the pathological findings of Claire's autopsy at the Inquest.**

(h, i & ii)

At the time of the Inquest I assumed that I had been the author of the final report. Also I had reviewed the case before the Inquest and agreed with conclusions in the report. I often attended and gave evidence at Inquests relating to autopsies in which I was involved as a Senior Registrar. There is a list of doctors who have the ability to do this available from the Coroner. I was on this list of doctors.

(21) **Describe any discussions between 1996 and 2006 in relation to:**

(a) **any request/s for information relating to Claire's autopsy report or**

(b) **attending Claire's Inquest**

and state the date and nature of each conversation, the parties to each discussion and any record of each discussion.

(a&b)

As the autopsy was in 1996 and the Inquest was held in 2006 I do not recall the dates or times of any specific discussions or conversations.

III. ADDITIONAL QUESTIONS

(22) **Identify precisely on the attached copy notes and records from the Regional Neuropathology Service in relation to the autopsy carried out on Claire Roberts the entries that you made or which were made on your direction and state below and state:**

(a) **When each of the identified entries was made**

(b) **The source of the information recorded in the entry.**

The Provisional Anatomical Summary is signed by me, but I am not aware of its date. I have not seen any other documents signed by me.

(23) **In relation to the attached copy notes and records from the Regional Neuropathology Service relating to the autopsy carried out on Claire Roberts:**

(a) **Explain the name and purpose of the document at Ref: 090-054-178.**

This is a Day Book. It is a record of material that enters and leaves the laboratory.

(b) **Identify the persons who made the handwritten notes on Ref: 090-054-178 and their job title.**

There are two different handwritings and I do not know who they pertain to.

(c) **Explain the meaning and significance of the entry:**

"Brain Blocks X10 √ H+E Date In 28/11/96 Date Out 23/12/96". **(Ref: 090-054-178)**

(i) **Explain what *"H+E"* means.**

Tissue blocks of brain are taken and processed in the laboratory. From these blocks, glass slides of tissue are prepared. These are stained with chemicals to show the cells in the tissue. One of these stains is called H&E (haematoxylin and eosin).

(ii) **Explain where the brain blocks went *"in"* to on 28th November 1996, and why this was being done at this time.**

This refers to the date that they entered the laboratory. This is also the date of the brain cut which is performed in a room separate from the main laboratory. At the brain cut these tissue blocks were taken for further processing in the laboratory.

(iii) **Explain where the brain blocks went *"out"* to on 23rd December 1996, and why this was being done at this time.**

'Out' refers to when the slides (not blocks) were given to the pathologist.

(d) **Explain the meaning and significance of the entry:**

"Further blocks Dr.Mirk. 31.1.97" **(Ref: 090-054-178) and:**

(i) **Identify *"Dr.Mirk."*, his/her position at that time and his/her involvement and role in Claire's autopsy and autopsy report.**

Dr Mirakhur was the consultant in charge of the case and her role in the autopsy report has been discussed in previous answers.

(ii) **Explain what was happening to further blocks at that date and the purpose of this.**

It is quite often necessary to take further blocks of tissue from the brain after the initial blocks have been examined. This would be routine.

(iii) **Explain why this was being done at that time and identify who directed/requested that it be done.**

It would have been done at this time because the slides made from the initial blocks would have been reviewed by the pathologist. It is likely that was directed by Dr Mirakhur.

(iv) **Explain who cut the blocks and when this was done.**

It is likely that further blocks were cut by Dr Mirakhur on 31/1/1997.

(e) **Explain the meaning and significance of the entry:**

"1Mamillary bodies HE

4 Brain"

Urgent for NSU

EB'S out to Dr.Mirk. *Date Out 6.2.97"*(Ref: 090-054-178) and:

(i) **Explain what *"Mamillary bodies"*areand why a block was being taken from there.**

The mamillary bodies are part of the brain. These blocks were necessary to look at this particular anatomical area.

(ii) **Explain what *"HE"*is, when this was done and by whom.**

HE = H&E, see answer 23, C (i). This would have been done by the biomedical scientist, name not recorded.

(iii) **Explain what *"4 Brain"* is and explain why 4 blocks were being taken from there.**

4 Brain refers to 4 further blocks of brain tissue. As indicated above it would be routine to take further blocks of brain tissue after the initial analysis was done.

(iv) **Explain what *"NSU"*is.**

See answer 5a above (neuroscience meeting).

(v) **Explain why it was *"Urgent"***

As this is not my handwriting and this was a case from 1996 I cannot comment on the specifics of why this was urgent. However it is likely that the case was to be discussed at the Neuroscience Meeting (NSU) and the slides were being prepared in anticipation of this meeting.

(vi) **Explain what *"EB'S"*are.**

This refers to Extra Blocks.

(vii) **Explain why *"EB'S"* were going *"out to Dr.Mirk"* at that time, identify who directed that this be done, and state the purpose of this and what was going to be done to the *"EB'S* .**

As indicated above it is often the practice to take extra blocks of tissue once the initial blocks have been examined. This was directed by Dr Mirakhur.

(f) **Explain the meaning and significance of the entry:**

"Cord x 2 *Date In 1/5/97* *Date Out 23/5/97"*(Ref: 090-054-178) and:

(i) **Explain what *"Cord x 2"*is and what was being done with it.**

(ii) **Explain why this was being done at that time and identify who directed that it be done.**

(iii) **Explain the meaning of *"Date In 1/5/97"*and why this was being done at that time, particularly after the autopsy report had been furnished.**

(iv) **Explain the meaning of *"Date Out 23/5/97"* and why this was being done at that time,particularly after the autopsy report had been furnished.**

(i-iv)

This was a brain only autopsy with no record of the spinal cord having been retained. I did not make this entry and there is no indication that I was present at this time. It is difficult to be entirely certain but it is likely to be a mistaken entry in the day book.

(g) **Explain the name and purpose of the documents at Ref: 090-054-179 and 180.**

These are laboratory Daybooks. When a case comes to neuropathology its details are recorded in these books.

(h) **Identify the persons who made the handwritten notes on Ref: 090-054-179 and 180 and their job title.**

The name Li Ping is attached to these pages. She was a medical laboratory assistant in the department at the time. It is possible that this is her handwriting.

(i) **Explain the meaning and significance of the entry:**

"Specimen Received

Brain only. 1 piece for

snap Frozen 2 Blocks

1 piece for EM *Date in 24/10/96"*(**Ref: 090-054-179**)

(i) **Explain the meaning of *"snap"***

This is a method of freezing tissue.

(ii) **Explain the meaning of *"1 piece for snap Frozen 2 Blocks"***

One single piece of tissue was divided into two smaller pieces for freezing.

(iii) **Explain what was done with the sample *"for snap Frozen 2 Blocks"*, whether it was subject to any further examination or tests, and if so the results of that test/examination, and the location of this sample now.**

To my knowledge it was not subjected to any further examination. This tissue was present when checked in 2007. I understand that the tissue was irrevocably damaged by a number of freezer failures between 2007 and 2009.

(iv) **Explain the meaning of *"EM"*.**

EM refers to Electron Microscopy.

(v) **Explain the meaning of *"1 piece for EM"*.**

One piece of tissue was taken for electron microscopy.

(vi) **State whether the sample was subject to *"EM"*, and if so, when, what was the result or outcome of *"EM"*analysis and identify the record thereof. If not, explain why not.**

The sections taken for electron microscopy were processed to thin sections (semi-thins). No further analysis was performed.

(j) **Explain the meaning and significance of the entry:**

M.L.S.O.	*Li Ping*
Diagnosis	*viral o Encephalitis Epilepsia*
Cut up - Notes	*Cut up 28/11/96 - Dr. Herron - Blocked*
Store/out etc.	*KPH 28/11/96*
	Out 24/4/97" **(Ref: 090-054-180)**

(i) **Explain the meaning of *"M.L.S.O"***

This is a Medical Laboratory Scientific Officer.

(ii) **Identify the position of *"Li Ping"*and his/her involvement in Claire's autopsy.**

Li Ping was a medical laboratory assistant. She made the entry relating to Claire's autopsy in the neuropathology day book.

(iii) **State the basis of the recorded diagnosis on this form.**

As the case was in 1996 and I did not make this entry I cannot comment on the source of this information.

(iv) **Explain the meaning of *"viral o Encephalitis Epilepsia"*, and clarify the diagnosis recorded.**

As I did not make this entry I cannot comment further.

(v) **Explain the meaning of *"Cut up - Notes"***

The material retained and other details regarding the case are recorded in this Day Book as Cut up Notes.

(vi) **Explain the meaning of *Cut up 28/11/96 - Dr. Herron - Blocked"*, explain what was done, when and identify who did it and why it was being done on *"28/11/96"*.**

This refers to the brain cut.

(vii) **Explain the meaning of*"Store/out etc."***

This refers to what happens to the tissue when the case is complete.

(viii) **Explain the meaning of *"KPH 28/11/96"* and explain what happened on *"28/11/96"*, who was involved and why it happened then.**

This refers to the brain cut. KPH is an abbreviation for Keep Pending Histology.

(ix) **Explain the meaning of *"Out 24/4/97"* and explain what happened on *"24/4/97"* and why it happened then, particularly after the autopsy report had been furnished, what was done with the sample then and where the sample is now.**

This refers to tissue disposal. I am not familiar with the methods of disposal in 1997.

(x) **If samples were stored, please state how they were stored, and in particular whether they were stored in formalin.**

The brain tissue that was not processed into blocks and slides is called wet tissue. From these documents it appears it was disposed of in 1997. As these wet tissues were disposed of on 24/4/1997 they would not have been stored beyond this time. However tissue slides and blocks were stored beyond this time as would be routine.

(k)**Explain the name and purpose of the document at Ref: 090-054-182.**

This is a further page from the Daybook. This would have been the page facing document 090-054-181.

(i) **Identify the persons who made the handwritten notes on Ref: 090-054-182 and their job title.**

I do not know whose handwriting this is.

(l) **Explain the meaning and significance of the entry:**

"Stained slides →D. Sgt. B. Cross for referral to Dr. B. Harding

Copies of Parental Consent and Coroners Information rec'd.

Copies of Inventory - *B. Cross*

- *Sp. Case file*
- *Archivist"*(Ref: 090-054-182)

(i) **Identify by whom this note was made and state when it was made.**

I do not know whose handwriting this is.

(ii) **Explain what the *"Inventory"* is and furnish a copy thereof.**

An inventory is a list of the material in any individual case. This will be provided.

(iii) **Explain what the *"Sp. Case file"* isand furnish a copy thereof.**

In neuropathology any case that leaves the department is recorded in a special case file. This runs to several thousand pages and is not in one location. The special case file is filed with each individual case. The entries relating to Claire Roberts can be made available.

(iv) **State whether Claire's case was included in the *"Sp. Case file"* and if so, state when and the reasons why.**

Yes, for the reasons in answer iii above.

(v) **Explain the meaning and purpose of *"Archivist"* and identify that person by name.**

The archivist in pathology is the person who traces the comings and goings of tissue, slides etc. Catherine Kilpatrick has only been appointed recently and was not a member of staff in 1996/7

(m) **In relation to the *"Autopsy Request Form"* :**

(i) **State the number of pages in this form and provide a complete double-sided copy of the complete form.**

Four pages. This will be provided.

(ii) **Explain the meaning of *"the requesting doctor"* and state whether that doctor is normally the Consultant who was responsible for the paediatric patient. If not, then identify who is normally *"the requesting doctor"*.**

The requesting doctor is the doctor who requests the autopsy. It would normally be a consultant.

(iii) **State whether *"the findings of the autopsy"* were *"telephoned"* to Dr. Steen, and if so, state when this was done, by whom and identify any note, record or document relating thereto.**

As this case was in 1996 I have no personal recollection as to whether this was done or not.

(iv) **State the purpose of *"the findings of the autopsy"* being *"telephoned"* to the requesting doctor in paediatric autopsy, when this is usually done, by whom and what note or record is made thereof.**

Quite often the results will be telephoned to the requesting doctor. In Claire's case there would have been little to discuss at the initial stage. This is because the brain needed to be retained for further examination before any result would be available. This information may or may not have been telephoned at the time of the autopsy or later.

(v) **State whether you took any steps to check or ascertain the accuracy and impartiality of the information in the autopsy request form, and in particular the information relating to Claire's clinical presentation, history, diagnosis and clinical problems on that form. If so, describe those steps, when you took them and what the outcome was. If not, explain why not.**

As this case was in 1996 I have no recollection whether any steps were taken.

(vi) **Identify the chart referred to in the form in *"INVESTIGATIONS: See chart"* and furnish a copy thereof.**

As this case was in 1996 I have no recollection as to what chart this refers to. It may or may not refer to the medical notes. If it does refer to the medical notes these are not in my possession. I understand they are with the Trust litigation office.

In relation to the autopsy report at Ref: 090-054-186 to 188:

(vii) **Identify the person who made the handwritten notes on and amendments in this report and state when they were made.**

Dr Mirakhur, I cannot comment on when they were made.

(viii) **Explain the meaning of *"CODES".***

Pathological material may be coded in a number of ways. The most common method of coding for pathological material is called SNOMED coding.

(ix) **State whether you were involved in the drafting or preparation of this report, and if so describe your involvement and the dates thereof.**

As this case was in 1996 I have no recollection of my involvement in the drafting and preparation of this report.

(n) **In relation to the autopsy report at Ref: 090-054-190 to 192:**

(i) **Explain the meaning, significance of each reference and the site to which it refers:*"T-A0100, M-01000, D4-00000, M-40000, D4-41720"* (Ref: 090-054-190).**

These are SNOMED codes. T-A0100 = Brain. M-01000 = Pathological Abnormality Present. D4-00000 = Congenital/Genetic Chromosomal Abnormality, M-40000 = Reactive Change, D4-41720 These codes have been superseded and I cannot trace the meaning of this code at present

(ii) **Explain the reason why this report is dated *"25/10/96"* and *""11/2/97"* and what occurred on each date in relation to the report and autopsy.**

These refer to secretarial typing dates.

(iii) **State whether you were involved in the drafting or preparation of this report, and if so describe your involvement and the dates thereof.**

As this case was in 1996 I have no recollection of my involvement in the drafting and preparation of this report.

(o) **In relation to the autopsy report at Ref: 090-054-193 to 195:**

(i) **State whether you were involved in the drafting or preparation of this report, and if so describe your involvement and the dates thereof.**

As this case was in 1996 I have no recollection of my involvement in the drafting and preparation of this report.

(p) **In relation to the document at Ref: 090-054-196:**

(i) **Explain the name and nature of this document.**

This is a list of material that was held in a freezer in the department

(ii) **Identify the entries relating to Claire Roberts and the person who made those entries and the date when they were made.**

I am not able to say who made the initial entries (three columns on the left). The entry on the right Checked JM 6/3/07 is by John Murray, a Biomedical Scientist.

(iii) **Explain the meaning of *"Type Frozen SNAP"***

There are different methods of freezing tissue. The most rapid of these is called snap freezing.

(iv) **Explain the meaning and significance of *"checked JM 6/3/07"*.**

John Murray checked that tissue was present on 6/3/2007.

(v) **Identify *"JM"* and their job title and explain what was being checked, and why it was being checked on *"6/3/07"*.**

The freezer was checked as we were preparing an inventory of material at this time. It was checked by John Murray, Biomedical Scientist.

(q) **Explain why there are copies of 4 different versions of Claire's autopsy report held in the notes and records relating to Claire's autopsy.**

Any autopsy report may go through a number of drafts before a final report is issued. It appears these are drafts of the final report.

FURTHER INFORMATION

Research papers referred to in deposition

Porcine rubulavirus LPMV RNA persists in the central nervous system of pigs after recovery from acute infection.
Wiman AC, Hjertner B, Linné T, Herron B, Allan G, McNeilly F, Adair B, Moreno-López J, Berg M.
J Neurovirol. 1998 Oct;4(5):545-52

New form of autosomal-recessive axonal hereditary sensory motor neuropathy.
Eckhardt SM, Hicks EM, **Herron B**, Morrison PJ, Aicardi J.
Pediatr Neurol. 1998 Sep;19(3):234-5

Apoptosis in measles virus-infected human central nervous system tissues.
McQuaid S, McMahon J, **Herron B**, Cosby SL.
NeuropatholApplNeurobiol. 1997 Jun;23(3):218-24.

A sequential study of experimental porcine paramyxovirus (LPMV) infection of pigs: immunostaining of cryostat sections and virus isolation.

Allan GM, McNeilly F, Walker I, Linne T, Moreno-Lopez J, Hernandez P, Kennedy S, Carroll BP, **Herron B**, Foster JC, Adair B.
J Vet Diagn Invest. 1996 Oct;8(4):405-13.

An 18 week fetus with Elejalde syndrome (acrocephalopolydactylyous dysplasia).
Nevin NC, Herron B, Armstrong MJ.
ClinDysmorphol. 1994 Apr;3(2):180-4. Review

Amniotic band syndrome mimicking anencephaly
Herron B and others. Presented to Ulster society of Obstetrics and gynaecology 1992
Brian Herron

THIS STATEMENT IS TRUE TO THE BEST OF MY KNOWLEDGE AND BELIEF

Signed: **Brian Herron** **Dated: 16/5/12**

328

Northern Ireland Regional Neuropathology Service

Case Review Records

Name	Lab No	Hosp No
CLAIRE ROBERTS	NPPn 114/96	

Date	Comments
21/3/07	SEE COVERING LETTER
29/4/08	All slides returned from Det Sgt Cross via PSNI Constable
	Recd 23 Immune Slides 4 Semi thins 44 H+E Stained Slides (incls Levels + Duplicates)

HER MAJESTY'S CORONER

DISTRICT OF GREATER BELFAST

John L Leckey LL.M.
H.M. Coroner
Coroner's Office
Courthouse
Old Town Hall Building
80 Victoria Street
Belfast BT1 3GL
Northern Ireland

Telephone: (028) 9072 8202
Fax: (028) 9072 4559
E. mail: jleckey.rcj@[redacted]

Dr Brian Herron
Consultant Neuropathologist
Institute of Pathology
Royal Group of Hospitals Trust
Grosvenor Road
BELFAST
BT12 6BA

9th February 2005

Dear Brian,

CLAIRE ROBERTS, DECEASED – NPPM 114/96

I have received your letter of 3rd February.

My understanding is that the team within the hospital which re-assessed the events leading to the death of Claire concluded that hyponatraemia was the underlying cause of the brain swelling.

As soon as I have received the report from Dr Bingham I will provide you with a copy and ask for your comments. You may have noticed the statement made by Mr John O'Hara QC who is conducting the inquiry into hyponatraemia related deaths that a fourth child has been identified. This child is Claire Roberts and it would seem that his inquiry will be widened to include an investigation into her death provided the report of Dr Bingham confirms that there is a fluid management issue. I have advised him that once I receive the report from Dr Bingham I will forward a copy to him. It is my intention to hold an inquest regardless of the inquiry. I would anticipate that you will be asked to give evidence to the inquiry in relation to your post-mortem examination.

Yours sincerely

J L LECKEY
HM CORONER FOR GREATER BELFAST

The ROYAL HOSPITALS

N.I. Regional Neuropathology Service
Institute of Pathology
Royal Group of Hospitals Trust
Grosvenor Road
Belfast BT12 6BA
Tel: 02890 240503 Ext: [redacted]
Tel: [redacted] (direct line)
Fax: 02890 438024
Meenakshi.mirakhur@[redacted]
Brian.herron@[redacted]

3rd February 2005

Mr J L Leckey LL.M.
HM Coroner for Greater Belfast
Coroner's Office
Courthouse
Old Town Hall Building
80 Victoria Street
Belfast BT1 3GL

Dear John

Re: Claire Roberts, Deceased – NPPM 114/96

Thank you for your correspondence.

I have reviewed the pathological findings in the case. It seems that Mr Walby has referred the case to you because there is indication that hyponatraemia may have played a part in the death. The cerebral oedema (brain swelling) that was present may have many causes, one of which is hyponatraemia. The autopsy did not exclude this as a cause of the brain swelling nor did it show any specific findings (structural changes) to make the diagnosis of hyponatraemia.

I am unclear from the letter as to whether it is thought that the hyponatraemia was a primary factor in this case, ie caused the brain swelling, or was secondary to the brain swelling..

I understand that this case has gone for expert review and I would be happy to be of any further assistance.

Yours sincerely

Brian Herron
Consultant Neuropathologist

HER MAJESTY'S CORONER

DISTRICT OF GREATER BELFAST

John L Leckey LL.M.
H.M. Coroner
Coroner's Office
Courthouse
Old Town Hall Building
80 Victoria Street
Belfast BT1 3GL
Northern Ireland

Telephone: (028) 9072 8202
Fax: (028) 9072 4559
E. mail: jleckey.rcj@[redacted]

Dr Brian Herron
Consultant Neuropathologist
Department of Pathology
Royal Group of Hospitals
Grosvenor Road
BELFAST
BT12 6BA

21st December 2004

Dear Brian,

CLAIRE ROBERTS, DECEASED – NPPM 114/96

I am enclosing a copy of a letter I have received from Peter Walby.

I have a copy of your post-mortem report which I understand was limited to an examination of the brain only.

In view of what has now emerged I should be grateful for any additional comments you may wish to make.

Yours sincerely

J L LECKEY
HM CORONER FOR GREATER BELFAST

Enc

STATEMENT OF WITNESS

STATEMENT OF: ALAN JOHN ROBERTS
Name Rank

AGE OF WITNESS (if over 18 enter "over 18"): 14/9/58

TO BE COMPLETED WHEN THE STATEMENT HAS BEEN WRITTEN

I declare that this statement consisting of pages, each signed by me is true to the best of my knowledge and belief and I make it knowing that, if it is tendered in evidence at a preliminary enquiry or at the trial of any person, I shall be liable to prosecution if I have wilfully stated in it anything which I know to be false or do not believe to be true.

Dated this 16 day of MARCH 2007

SIGNATURE OF MEMBER by whom statement was recorded or received

SIGNATURE OF WITNESS

WILLIAM R. CROSS.
PRINT NAME IN CAPS

I am the father of Claire Roberts who was born on 10th January 1987 and passed away on 23 October 1996 at the Royal Belfast Hospital for Sick Children. I consent to the Police Service of Northern Ireland obtaining all tissues and samples held at the Royal Group of Hospitals or the State Pathology Department which relate to the autopsy conducted on Claire. I also consent to any medical or nursing professional assisting the PSNI in providing any other material, report, or statement relevant to the enquiries by the PSNI into the circumstances of Claire's death. I also consent to the PSNI obtaining a full copy of Claire's medical notes, or the originals of the same.

Form 38/36
(Lined)

PB 5/02

SIGNATURE OF WITNESS:

Murray, John

From: Billy.Cross@[redacted]
Sent: 20 March 2007 12:50
To: Murray, John
Subject: Claire Roberts

Consent.doc (49 KB)

John,

Attached is consent statement and copy of email from Mr Lecky.

If you can phone me as soon as you are in a position to release the material I will have it collected. I will have to take a short statement from the person handing the material to me.

Thanks

Billy Cross
DSgt
[redacted]

<<Consent.doc>>

Sir,

Noted, thank you.

Billy Cross
DSgt

-----Original Message-----
From: Leckey Mr, Senior Coroner [mailto:jleckey.rcj[redacted]
Sent: 06 March 2007 16:00
To: CROSS Billy
Subject: RE: Request for Authorisation (Claire Roberts dec'd)

Dear Sergeant,
This is to confirm our telephone conversation this afternoon. I have no legal authority to provide you with such authorisation as the cause of death has been established and an inquest has been held. You will have to approach Claire's parents for the necessary authorisation.

John Leckey
-----Original Message-----
From: Billy.Cross@psni.pnn.police.uk [mailto:Billy.Cross@[redacted]
Sent: 06 March 2007 15:54
To: Leckey Mr, Senior Coroner
Subject: Request for Authorisation

Dear Mr Coroner

Please find attached a letter, hard copy is in the post today. If you grant this authorisation I will have it collected by hand if your office rings me on 07802926197.

Thank you

Billy Cross
DSgt

<<HM Coroner3-04-06.doc>>

****

Kilpatrick, Catherine

From: Murray, John
Sent: 06 September 2011 16:06
To: Kilpatrick, Catherine
Subject: Recalls from Oasis

Categories: REQUESTS

Hi Tammy,

Would you be kind enough to recall the following material from Oasis: -

- The PM blocks of NPPM 114/96 which according to my database are in N'Path box 144 (Old Ref No. F63121)
- The PM slides of the same case i.e. NPPM 114/96 which are in filing cabinets I sent for storage.

Dr Herron needs these for a case he is involved with which is part of a judicial review.

Many thanks

John

22 slides (8 bis)
&
x 2 boxes.

copy of signed letter given to DSgt Cross by Dr Herron.

N.I. Regional Neuropathology Service
Institute of Pathology
Royal Group of Hospitals Trust
Grosvenor Road
Belfast BT12 6BA
Tel: 02890 240503 Ext: [redacted]
Tel: [redacted] (direct line)
Fax: 02890 438024
Brian.herron@[redacted]

20th March 2007

FAO:- Detective Sgt Billy Cross

Dear Detective Sgt Cross

RE: Claire Roberts NPPM 114/96

I release to your care the following:

32	H&E Stained slides.
23	Immunohistochemically Stained slides.
4	Semi thins

We still have a very small amount of tissue in a freezer and if this is required we need to make alternative arrangements as this would thaw out during normal transport. We also have 16 paraffin blocks that can be collected either now or at a later date.

Yours sincerely

Dr B Herron
Consultant Neuropathologist

Enc.

Belfast Health and Social Care Trust

N.I. Regional Neuropathology Service
Institute of Pathology
Royal Group of Hospitals Trust
Grosvenor Road
Belfast BT12 6BA
Tel: 02890 240503 Ext: [redacted]
Tel: [redacted] (direct line)
Brian.herron@[redacted]

25TH August 2011

Mr John Leckey
HM Coroner for Northern Ireland
Mays Chambers
73 May Street
Belfast BT1

Dear Mr Leckey

RE:- Claire Roberts DOB: 10/1/1987, DOD: 23/10/1996, NPPM 114/96

As you know there is ongoing enquiry into the hyponatraemia related deaths and Claire Roberts is one of the cases under investigation.

I remember there was an inquest into this death held by yourself. I have been asked to confirm that the slides and tissue blocks relating to this child have been retained and are secure in our Department. I wonder if you could clarify if there has been any instruction from your office regarding these tissues after the inquest was complete. My understanding is that once the inquest is complete and you have given guidance on the further use or disposal of these tissues they leave your jurisdiction and come back to my jurisdiction, but you may also be happy to clarify that point.

Yours sincerely

Dr Brian Herron
Consultant Neuropathologist

Copy to: **Dr *P Walby, Litigation Office, RVH***

Journal of NeuroVirology (1998) 4, 545–552

http://www.stockton-press.co.uk/jnv

Porcine rubulavirus LPMV RNA persists in the central nervous system of pigs after recovery from acute infection

Ann-Christin Wiman[1], Bernt Hjertner[1], Tommy Linné[1], Brian Herron[2], Gordon Allan[3], Francis McNeilly[3], Brian Adair[3], Jorge Moreno-López[1] and Mikael Berg[1,4]

[1]*Department of Veterinary Microbiology, Section of Virology, Unit of Molecular Virology, Swedish University of Agricultural Sciences, Biomedical Centre, Box 585, S-751 23 Uppsala, Sweden;* [2]*Department of Neuropathology, Royal Group Hospitals Trust, Belfast BT12 6BA, Northern Ireland;* [3]*Department of Agriculture for N Ireland, Veterinary Sciences Division, Stoney Road, Stormont, Belfast BT4 3SD, Northern Ireland;* [4]*Department of Veterinary Microbiology Section of Immunology, Swedish University of Agricultural Sciences, Biomedical Centre, Box 588, S-751 23 Uppsala, Sweden*

In order to study persistence of the porcine rubulavirus LPMV, we examined tissue samples collected from pigs 53 days after experimental infection. These pigs survived the initial infection and could clinically be considered to have recovered from the infection. Two of the pigs used in this study were chemically immunosuppressed during the last 4 days before necropsy. No infectious virus or viral antigen could be detected in any tissue using standard methods for virus isolation and detection. However, the presence of viral genomic RNA and mRNA could be demonstrated in the mid brain of the convalescent pig using an optimised RT-nested PCR. Mid brain, forebrain and lung were all shown to contain LPMV RNA in the immunosuppressed convalescent pigs. In addition we examined the P-gene editing in the recovered pigs and conclude that the viral genome is transcriptionally active in these pigs. The relevance of the persistence of LPMV for maintenance and spread within and/or between pig populations is discussed.

Keywords: paramyxoviridae; rubulavirus; persistent infection; P-gene editing

Introduction

Porcine rubulavirus LPMV (La Piedad Michoacan Mexico Virus), the causative agent of 'blue eye disease' in pigs, is a member of the paramyxoviridae family (Rima *et al*, 1995). Infection of pigs with LPMV causes encephalitis, pneumonia, and corneal opacity. In general the disease is more severe in young piglets (Stephano *et al*, 1988). LPMV has been analysed in great detail at the molecular level. The whole 15 kb genome has been sequenced and phylogenetic analysis has revealed the closest relatives to be mumps virus and simian virus 5 (Berg *et al*, 1991, 1992, 1997; Linné *et al*, 1992; Sundqvist *et al*, 1992; Svenda *et al*, 1997, 1998). Furthermore, the expression of the P-gene has been analysed. LPMV expresses the P-gene in a similar way to mumps virus, editing being necessary for expression of the P protein (Berg *et al*, 1992).

Correspondence: M Berg
Received 25 February 1998; revised 8 June 1998; accepted 30 June 1998

A detailed sequential study of virus distribution in tissues of pigs experimentally infected with LPMV has recently been reported (Allan *et al*, 1996). Pigs inoculated at 3 days of age with LPMV suffered from severe clinical signs and by day 8 after infection they were either dead or moribund. In contrast, pigs inoculated with virus at 17 days of age showed only mild clinical signs. Virus could be demonstrated in tissue samples from the respiratory tract and in the central nervous system (CNS) in pigs inoculated both at 3 and 17 days of age. However, differences in virus distribution within the CNS were demonstrated. In the younger pigs, virus was widespread throughout the CNS. In contrast, virus was restricted to the olfactory bulb and mid brain in the older pigs and no virus could be detected in any organ at 14 days after infection i.e. the infection seemed to be cleared.

Since the first outbreak of LPMV, the disease has spread throughout Mexico with disease outbreaks recorded in many states. Stephano *et al* (1988)

suggested that subclinically infected pigs could serve as a reservoir for the virus. They noted that when susceptible pigs were introduced into a farm operating on a continuous flow basis with a prior history of LPMV infection the newly introduced group showed clinical signs of LPMV infection. However, when a sentinel pig was introduced into closed herd 6–12 months after resolution of an outbreak the pig remained asymptomatic (Stephano, 1990). It is possible that persistence of LPMV in convalescent pigs could serve as a reservoir for the virus and may reactivate in a situation of natural immunosuppression and spread to other susceptible pigs. Whether LPMV can persist in convalescent pigs in nature has not yet been demonstrated.

The porcine rubulavirus LPMV can establish persistent infections in porcine kidney cells (Hjortner *et al*, 1997, 1998). The persistent infection has been shown to be stable for at least one year in continuous culture. Virus was observed to be released from the cells but at lower infectious titers than those released from lytically infected cells. Several large subgenomic RNAs were associated with the persistently infected cell, a common phenomenon in many persistent infections (Re, 1991). Reduced expression of several viral proteins was also noted.

The aim of this study was to document the possible persistence of LPMV and viral RNA in pigs after recovery from clinical illness. The results show that no infectious virus particles could be detected in any tissue. Virus specific RNA, both genomic and mRNA, could however be detected in brain tissue 53 days after infection. Viral RNA could also be detected in lung tissue when the convalescent pig was treated with the immunosuppressive drug cyclophosphamide 4 days prior to necropsy. We also show that mRNA editing of the P-gene occurs in the convalescent pigs, indicative of active viral transcription in these pigs.

Results

The aim of this study was to investigate whether LPMV can persist *in vivo* and if persistent virus can be reactivated by immunosuppression (CPA treatment) of pigs after recovery from clinical illness. To do so in a controlled way we examined tissue materials from three convalescent pigs 53 days after infection. Tissue samples from these animals were subjected to histophatological examination, virus isolation, immunofluorescent staining of cryostat sections and analysis for the presence of viral RNA.

Clinical signs and histopathology

The three pigs inoculated at 17 days of age with LPMV-84 virus all showed mild clinical signs typical of porcine rubulavirus infection, including mild respiratory and nervous signs between 4 and 10 days after inoculation. All three pigs recovered and were clinically normal from 12 days after infection. During the immunosuppression (CPA treatment) no clinical signs were noted in pigs R220 and R221. Histopathological examination revealed no evidence of active inflammation in tissue sections from pig R219. Pig R220 and R221 however showed active inflammation, involving leptomeninges and brain tissue (meningoencephalitis). The brain areas affected included the olfactory system and adjacent frontal lobe and also mid brain and pons. The inflammatory response consisted of perivenous lymphocytic cuffing, neuronophagia and microglial nodule formation predominantly in the grey matter (Figure 1). This type of inflammatory response is characteristic of a viral infection in the CNS. The lesions are similar to those observed in the acutely infected pig. However, the distribution of the inflammatory response was different. In the acutely infected animal the inflammation was predominantly in the nasal submucosal tissue and adjacent olfactory areas. In the convalescent CPA treated pigs (R220 and R221) the inflammation is also present in brainstem structures.

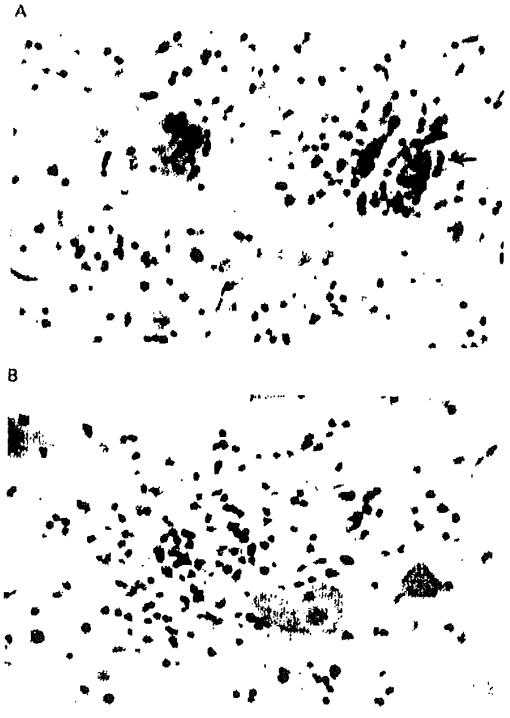

Figure 1 (a) Section through mid brain showing areas of active inflammation. The inflammatory infiltrate (arrow) is composed of lymphocytes located perivascularly ×2.5 magnification. (b) Brain stem nuclei with adjacent lymphocytic infiltrate ×2.5 magnification.

Absence of infectious virus and viral antigen in convalescent pigs

Virus isolation has previously been conducted on acutely infected pigs, infected at 17 days of age. Virus could be recovered from the CNS and this was restricted to the mid brain and olfactory bulb (Allan *et al*, 1996). However, in the convalescent pigs (R219, R220 and R221) tested in this study, no infectious virus could be detected in any tissue, including mid brain, forebrain and lung. The rectal and nasal swabs taken daily from day 49 after infection until necropsy also tested negative for infectious virus. In the acutely infected control pig (R233) virus was recovered from respiratory tract tissue and was also seen to be widespread in the CNS. No virus was detected in the uninfected control pig (R400). In addition immunofluorescent staining of cryostat sections was performed on the same tissues as were used for virus isolation, and no viral antigen could be detected in the recovered pigs or in the uninfected control pig. Viral antigen could be detected in the respiratory tract and in the CNS of the acutely infected control pig.

Presence of LPMV specific RNA in convalescent pigs

RT-nested PCR was performed using 1 μg of total RNA isolated from mid brain, forebrain and lung as template. Tissues which tested negative were retested with 10 times more (10 μg) of total RNA. In the P-gene RT-nested PCR analysis we included assays using appropriate primers for selection of LPMV genomic RNA (P genomic primer) or LPMV mRNA (oligo dT primer) in the RT reaction.

In the acutely infected control pig (R233), both NP- and P-gene specific LPMV RNA could be demonstrated in the organs tested (Figure 2 lanes 18 and 20, Figure 3b lanes 13–15, Figure 3c lane 6). In this pig nested PCR was not necessary and 1 ng RNA was enough to detect LPMV specific RNA (data not shown). No LPMV specific RNA could be detected in the uninfected control pig (R400) (Figure 2 lanes 11, 21, 22, Figure 3a lane 3, Figure 3b lanes 3–5 and Figure 3c lane 3). In the convalescent pig (R219), P gene specific LPMV mRNA could only be detected using 10 μg total RNA from mid brain (Figure 3a lanes 4,5) but not RNA from forebrain or lung (Figure 3b lane 6, Figure 3c lane 4). None of the tissues used in the assay tested positive for NP gene specific RNA (Figure 2 lanes 7, 8, 19). However, in convalescent pigs which had been immunosuppressed with CPA (R220 and R221) both NP and P gene specific LPMV RNA could be detected in mid brain and forebrain (Figure 2 lanes 6, 9, 10, Figure 3a lanes 7–9 and Figure 3b lanes 7–12). In one of the CPA treated convalescent pigs (R220) LPMV RNA was demonstrated in lung tissues as well, but the amount of RNA present was low (Figure 2 lane 23, Figure 3c lane 5). A summary of all these results is presented in Table 1.

The results presented here clearly demonstrate that both genomic RNA and mRNA are present in the brain of convalescent pigs. This indicates that LPMV can persist *in vivo*. Furthermore, the amount

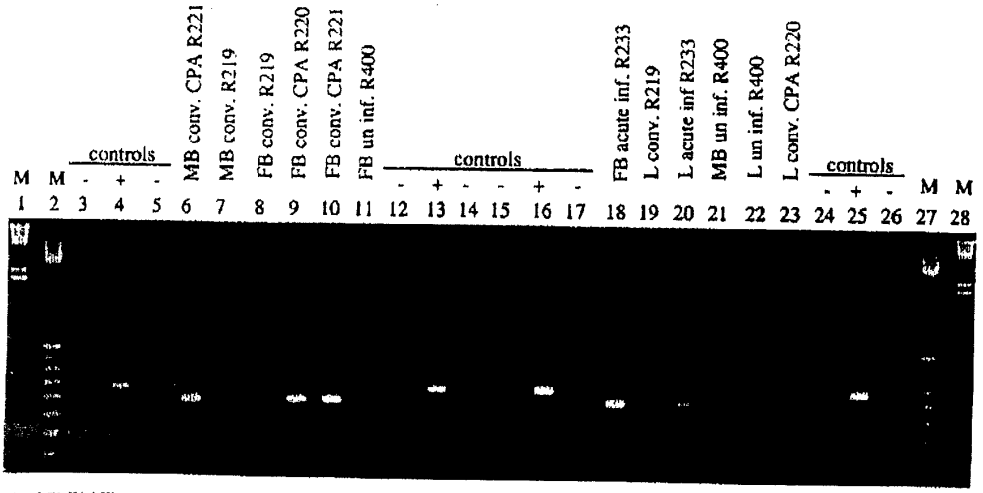

Figure 2 LPMV NP-gene specific RT-nested PCR products visualized by agarose gel electrophoresis. RT-nested PCR done with total RNA extracted from mid brain, forebrain and lung. Random hexamer primer are used in the RT reaction (lanes 6–11 and 18–23). M indicates 100 base pair (bp) ladder (lanes 2 and 27) and λ DNA *Hin*dIII digest (lanes 1 and 28). + indicates positive plasmid control (lanes 4, 13, 16 and 25). conv. indicates convalescent pig.

of detectable LPMV RNA increases in the brain of immunosuppressed pigs and is even detectable in the lungs of these animals.

Primer extension analysis demonstrates active transcription in convalescent pigs

Since some transcripts from the P-gene contain extra nontemplated nucleotides it is possible to assay for active transcription by identifying these mRNAs. The 298 base pair fragments shown in Figure 3a and b, were used in the primer extension assay. As expected, only the 28 nt product was seen to be present when the genome specific primer was used in the RT reaction (Figure 4a lanes 4–7). When oligo(dT) primer was used in the RT reaction, samples from the acutely infected control pig (R233) and the convalescent CPA treated pigs (R220 and R221) were shown to contain RNAs which gave both 28 and 30 nt products, as judged by comparison with the V and P plasmid controls (Figure 4a lanes 9–12). Surprisingly, the convalescent pig (R219) show only the extension product originating from a P specific transcript (Figure 4a lane 8). This is somewhat surprising, but may indicate that only a few molecules are being amplified in the RT-nested PCR, and that these organs contain quantities of LPMV RNA which are at the lower limit for detection by this method. Another explanation could be that the frequency of the P transcripts are much higher in this pig. To check this we tested mid brain from the convalescent pig (R219). This time we used an mRNA selection approach to increase the amount LPMV RNA/μg total RNA. We also tested mid brain from the convalescent CPA treated pig (R220). This time only the unedited transcript was detected in pig R219 (Figure 4b lanes 4, 5). However this mRNA selection approach may select the negative stranded genomic RNA since it can bind to the mRNA during the selection which will give a high background of genomic RNA.

This result endorses our view that the RNA amount is at the lower limit for detection. These observations further confirm that both genome RNA and mRNA of the P gene are present in the convalescent pigs, although in very small amounts. It also shows that mRNA editing of the P gene occurs, and that viral transcription is active.

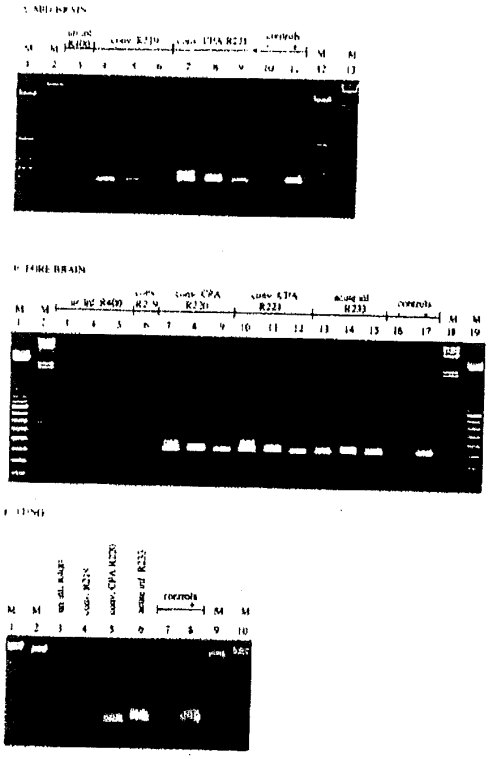

Figure 3 LPMV P-gene specific RT-nested PCR products visualized by agarose gel electrophoresis. conv. indicates convalescent pig. (a) RT-nested PCR done with total RNA extracted from mid brain. M indicates 100 base pair (bp) ladder (lanes 1 and 12) and λ DNA *Hind*III digest (lanes 2 and 13). Primers used in the RT reaction are; random hexamer (lanes 3, 4, 7, 10 and 11), oligo dT (lanes 5 and 8) and P347+ (lanes 6 and 9). (b) RT-nested PCR done with total RNA extracted from forebrain. M indicates 100 base pair (bp) ladder (lanes 1 and 19) and λ DNA *Hind*III digest (lanes 2 and 18). Primers used in the RT reaction are; random hexamer (lanes 3, 6, 7, 10, 13, 16 and 17), oligo dT (lanes 4, 8, 11 and 14) and P347+ (lanes 5, 9, 12 and 15). (c) RT-nested PCR done with total RNA extracted from lung. M indicates 100 base pair (bp) ladder (lanes 1 and 10) and λ DNA *Hind*III digest (lanes 2 and 9). Primer used in the RT reaction is random hexamer (lanes 3–8)

Discussion

New outbreaks of blue eye disease appear to emerge selectively in farms working with a continuous flow system suggesting that newly introduced pigs acquire the disease from pigs in the herd into which they are introduced (Stephano *et al*, 1988). This observation has led to the contention that a reservoir of LPMV is maintained in subclinically infected pigs. In view of a finding by Allan *et al* (1996) this seems unlikely. The study conducted by this group showed that no infectious virus could be isolated from day 14 post infection and onwards in LPMV infected pigs. An alternative explanation of the epidemiological data could be that persistently infected pigs which have recovered from the acute phase of infection act as a reservoir, shedding virus in situations of natural immunosuppression such as stress. The aim of this study was to elucidate whether the porcine rubulavirus LPMV could persist in pigs after full recovery from infection. In

three cases out of three, we have demonstrated that LPMV RNA persists in pigs for at least 53 days after infection. No infectious virus or viral antigen could be detected. Both genomic RNA and mRNA were detected at this time. In addition, RNA editing of the P gene was demonstrated, indicating that the viral genome was actively transcribed. According to these findings it seems likely that LPMV can persist at least in the form of viral genetic material, possibly without the production of infectious virus. This may be of importance for maintenance of the virus infection in the individual and could also have profound effects on the spread of virus within and/or between pig populations.

Results similar to these have been reported for Vesicular Stomatitis Virus, New Jersey strain (VSV-NJ) infections in hamster (Barrera and Letchworth, 1996) and in cattle (Letchworth *et al*, 1996). In hamsters, persistence of VSV RNA could be detected in the CNS 10–12 months after infection, but no infectious viral particles could be detected. In cattle, persistence of VSV RNA but not infectious viral particles could be detected in the tongue and in lymph nodes draining the tongue 5 months after infection. Our results agree well with these findings, indicating that persistence of viral RNA may be a fairly common feature after clearance of negative stranded RNA virus infections.

In a situation of immunosuppression, infectious virus could be produced and shed by persistently infected animals enabling infection of susceptible pigs. In our study, immunosuppression led to the detection of enhanced levels of LPMV RNA. Also, viral RNA could be detected in lung tissue. This indicates that a limited reactivation of the virus occurred. Immunosuppression of the pigs used in this study was carried out for a fairly short time, 4 days. Prolonged suppression might lead to further reactivation of the LPMV and the eventual production of infectious viral particles. Experiments utilising prolonged immunosuppression times will be of importance in elucidating whether recurring

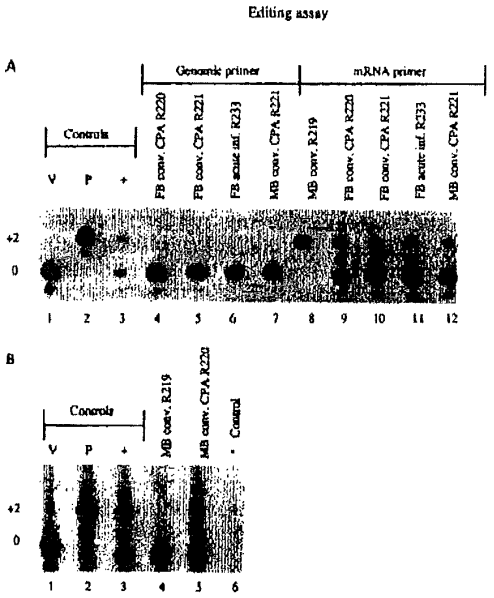

Figure 4 Primer extension analysis of RNA isolated from recovered and acute infected pigs. V indicates a plasmid control containing only V transcript cDNA (lane 1). P indicates a plasmid control containing only P transcript cDNA (lane 2). The V transcript is indicated with O. The P transcript is indicated with +2. conv. indicates convalescent pig. (a) Primers used in the RT reaction are: P-genomic (lanes 4–7) and oligo dT (lanes 8–12). (b) mRNA selected with oligo dT beads. RT reaction with random hexamer primer (lanes 3–6). Control indicates PCR product from the P-gene as a negative primer extension control.

Table 1. Summary of RT-nested PCR analysis of LPMV convalescent pigs

Animal	*Organ material*	*NP-gene PCR pd(N)[a]*	*pd(N)[a]*	*P-gene PCR oligo(dT)*	*genomic*
Convalescent CPA R221	Midbrain (1 μg RNA)	+	+	+	+
	Forebrain (1 μg RNA)	+	+	+	+
Convalescent CPA R220	Midbrain (1 μg RNA)	ND	+[b]	ND	ND
	Forebrain (1 μg RNA)	+	+	+	+
	Lung (10 μg RNA)	–	+	ND	ND
Convalescent R219	Midbrain (10 μg RNA)	–	+[b]	+	
	Forebrain (10 μg RNA)	–	–	ND	ND
	Lung (10 μg RNA)	–	–	ND	ND
Uninfected R400	Midbrain (10 μg RNA)	–	–[b]	ND	ND
	Forebrain (10 μg RNA)	–	–	–	–
	Lung (10 μg RNA)	–	–	ND	ND
Acute infected R234 (no nested PCR needed)	Forebrain (1 μg RNA)	+	+	+	+
	Lung (1 μg RNA)	+	+	ND	ND

[a]Primers used in reverse transcription. ND indicates not done. [b] indicates that the organ is tested with mRNA selected material only. [b]indicates that the organ was tested with both mRNA and total RNA selected material.

outbreaks of blue eye disease in farms with a continuous flow system, originate from persistently infected pigs.

Several factors are of importance in promoting the establishment of persistent infection of a cell. The virus has to assume a non-lytic mode of replication preferably in a long-lived cell which is not subjected to immune surveillance and escape the immune response (Randall and Russell, 1991). Neurons lack constitutive expression of MHC class I, and are comparatively insensitive to the induction of de novo expression of these molecules. Furthermore, they are very long-lived and thus meet both requirements for establishment of persistent infection (Sedgwick and Dörries, 1991). LPMV has been shown to infect neuronal cells by immunofluorescent staining of cryostat sections (Kennedy, S personal communication).

It has been shown that the highest titers of virus in acutely infected pigs with LPMV can be found in the mid brain (McNeilly *et al.* 1997). Therefore, we decided to assay for RNA in brain tissue of convalescent pigs as an initial step in characterisation of the persistent state. Other organs however could be more important in terms of virus shedding and spread to other animals. We therefore extended our study to include lung tissue, and one of the immunosuppressed pigs was shown to harbour viral RNA in the lung.

In conclusion, both LPMV genome and mRNA can be recovered in pigs after recovery from the acute phase of the disease, indicating persistent infection of these pigs. Whether these pigs can shed infectious virus remains to be shown.

Materials and methods

Virus

A cell culture strain of the porcine rubulavirus, designated LPMV-84, was used throughout this study. A virus pool was prepared. The infectious titer of this working pool was calculated to be $10^{7.36}$ $TCID_{50}$/0.1 ml. This virus pool was examined for evidence of contamination with pseudorabies virus, classical swine fever virus, and hemagglutinating encephalitis virus using direct immunofluorescence (IF) staining of acetone-fixed cell culture preparations. No evidence of contamination of the LPMV preparation with these viruses was detected.

Pigs

All of the pigs used in this study were obtained from a closed, minimal disease Large White breeder/finisher unit. The animals were shown to be free of porcine rubulavirus, pseudorabies virus, porcine parvovirus and classical swine fever virus by indirect immunofluorescence using sera from the pigs and cell cultures infected with either of the three viruses.

Three 17-day-old pigs (R219, R220, R221) were experimentally infected by intranasal and eyedrop routes with $10^{7.00}$ $TCID_{50}$ of the pool of LPMV-84 virus described above. They were euthanized after infection by intravenous barbiturate injection at 53 days after inoculation. R220 and R221 were chemically immunosuppressed by inoculation with cyclophosphamide (CPA) by the intraperitoneal route (30 mg/kg body weight) at 49 days after infection and again at 51 days after infection. CPA has been used as an immunosuppressant drug in pigs (Mackie, 1981). Pig R219 was not chemically immunosuppressed. Rectal and nasal swabs were taken daily from all three pigs from 49 days after infection until necropsy. Pig R233 was incoculated with the same virus pool and dose at 3 days of age and sacrificed when clinical signs of virus infection were evident (Allan *et al.* 1996). Pig R400 served as an uninoculated control for the duration of the experiment.

At necropsy, tissue samples listed in Allan *et al* (1996), were taken for histopathological examination, virus isolation, immunofluorescent staining of cryostat sections and analysis for the presence of viral RNA.

Histopathology

Immediately following death the brain and spinal cord were extracted and fixed in 10% formalin. The nasal mucosa and olfactory projection to the nasal area were extracted in continuity with the rest of the brain. Following fixation in formalin the tissues were dissected according to a fixed protocol. The brain stem and cerebellum were dissected from the brain and then the cerebral hemispheres were divided into 10 coronal slices from anterior to posterior (frontal to occipital). The brain stem and cerebellum were then sectioned from mid brain to medulla in transverse slices. Sections were taken from the cervical, thoracic and lumbar spinal cord. Each of these sections was stained with Haematoxylin and Eosin (H&E).

Virus isolation and immunofluorescent staining of cryostat sections

Virus isolation has been described earlier (Allan *et al.* 1996). Briefly, tissue samples were suspended in Minimal Essential Medium, centrifuged, and the supernatant was inoculated into PK-15 cell cultures and incubated for 6 days at 37 °C. The cell cultures were then freeze/thawed once and the cell lysates were inoculated into fresh PK-15 cell cultures, incubated and freeze/thawed again as described above. The resulting cell lysates were assayed for hemagglutination (HA) activity and by immunofluorescence (IF) using a hyperimmune antiserum to the LPMV-84 isolate prepared from a rabbit (Allan *et al.* 1996). Tissue samples for cryostat sectioning were processed and immunostained (McNeilly *et al.* 1991) using the same antiserum as above.

RT-nested PCR of LPMV RNA

Total RNA was purified using the RNaid® KIT (BIO 101), and polyA⁺ RNA was purified using the Quick Prep®Micro mRNA Purification Kit (Pharmacia Biotech) according to the manufacturer's protocol. Either 1 or 10 μg RNA in a volume of 9 μl was heat denatured (70°C, 5 min), and reverse-transcribed in 20 μl containing 4 μl 5 × RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 4 μl 2.5 mM dNTP mix, 1 μl 33 u/μl RNA guard (Pharmacia Biotech), 1 μl 200 u/μl moloney murine leukeamia virus reverse transcriptase (Promega) and 1 μl 20 pmol/μl primer (random hexamers, oligo(dT) or LPMV genome (P-gene) specific; 5'-GGCCCACCCTACACTCCCCA-3'). The mixture was incubated at 20°C for 5 min and then 37°C for 90 min. The reverse transcriptase was subsequently inactivated at 98°C for 5 min. Ten μl of this viral cDNA was then amplified by nested PCR. Primers for the LPMV virus NP gene were designed from the NP gene sequence (Svenda *et al*, 1998) and had the following sequence: external primers, 5'-ATTCTC-CTTGCCTGCTGCTGCTAT-3' (sense) and 5'-AGTG-CCAAGTATCGTGCCTGTTCA-3' (antisense); internal primers, 5'-TCCCCCGATGCCGATTATTGAC-3' (sense) and 5'-CCCCCTTCGAGCTGGATTCTC-3' (antisense). The final nested product was 375 bp (or 474 bp for the plasmid control). Primers for the LPMV virus P-gene extending over the editing site, were designed from the P-gene sequence (Berg *et al*, 1992) and had the following sequence: external primers, 5'-CCACTCCGAGGTTCATCATCGAC-3' (sense) and 5'-TGCGGCCCTCGATTGCTTTC-3' (antisense); internal primers, 5'-ATGAGGGC-GATCTGATGGCG-3' (sense) and 5'-ATCTCCGG-CACATTGAGGGC-3' (antisense). The final nested product was 298 bp.

The first PCR was carried out in a 50 μl solution containing 5 μl 10 × PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 0.01% gelatin), 3 μl 25 mM $MgCl_2$, 4 μl 2.5 mM dNTP mix, 0.2 μl 5 u/μl AmpliTaq® DNA Polymerase (Perkin Elmer), 1 μl 10 pmol/μl each of the external primers and 10 μl cDNA. The thermal cycling program consisted of: five cycles of 94°C for 1 min, 58°C (NP gene primer set) or 62°C (P gene primer set) for 1 min and 72°C for 2 min, followed by 30 cycles of 94°C for 1 min, 53°C (NP gene primer set) 58°C (P-gene primer set) for 1 min and 72°C for 2 min, followed by 72°C for 10 min. For the nested PCR, 5 μl of the first PCR reaction was transferred to a nested PCR mix in a 50 μl solution as above except that internal primers were used. The thermal cycling program consisted of: 25 cycles of 94°C for 1 min, 53°C (NP gene primer set) 58°C (P gene primer set) for 1 min and 72°C for 2 min, followed by 72°C for 10 min.

To be able to differentiate between the positive control and positive samples, we constructed a clone which gave a longer PCR product (474 bp) compared to the wild type (375 bp), and had a unique restriction site, enabling us to specifically digest the control PCR product. The polylinker in the pUC-19 plasmid was amplified by PCR using M13 universal and reversal primers (Pharmacia). The resulting PCR product was blunt and ligated into a filled in *NsiI* site in a pUC19-NP-gene plasmid.

Total RNA extracted from lytically infected cells was included in each set of experiment as a positive control. In addition, we also included several negative dH_2O controls, to demonstrate that our routines were free from contamination. The final PCR product was analysed by agarose gel electrophoresis (1.5% gel), and visualized by ethidium bromide staining. Some of the PCR products were extracted from agarose gel slices using the QIAEX DNA Gel Extraction Kit (QIAGEN), according to the manufacturer's protocol, and subsequently used in primer extension analysis. To avoid contamination of the PCR reactions, careful routines for each step were set up, with separate rooms for: RNA extraction, RT-reaction, first PCR, nested PCR and PCR product analysis. UV-light and 10% chlorine solution were used after each step to destroy possible contaminants.

Quantification of the NP specific RT-nested PCR

A pcDNA3 plasmid construct containing the NP gene (pcDNA3-NP) was digested with *XhoI*. RNA was transcribed from the cleaved plasmid using T7 RNA Polymerase according to the protocol supplied by the manufacturer (Biolab). The reaction mixture was incubated at 37°C for 2 h, then treated with DNase I to remove the DNA template. After DNase I treatment the RNA was extracted with phenol-chloroform and then precipitated. The washed and dried RNA pellet was redissolved in diethyl pyrocarbonate treated water and extracted using the RNaid® KIT. The RNA was quantified spectrophotometrically at absorbance 260 nm, and a portion was analysed on an agarose gel to check the quality. To confirm that RNA and not original template DNA was amplified, the 'synthetic' RNA was assayed by nested PCR without reverse transcription. Extracted total RNA from mid brain from the negative control pig at a concentration of 0.1 μg/μl was used as carrier RNA to make 10-fold dilution series with the 'synthetic' RNA, which was subsequently used in the RT-nested PCR. Viral RNA could be detected at a dilution of 10^6 (1000 copies) but not 10^7 (100 copies). The sensitivity of the P gene RT-nested PCR was not tested, but our experiments indicate that it is at least as sensitive as the NP gene PCR or possibly slightly better.

Primer extension analysis

The primer extension analysis has been described previously (Berg *et al*, 1992). Briefly, the extracted PCR product was denatured with NaOH and then precipitated and annealed with a ^{32}P-5' labelled

primer complementary to sequences immediately downstream of the editing site of the P-gene of LPMV. The extension was done with T7 DNA polymerase (Pharmacia Biotech) and a label mix consisting of dCTP, dGTP, dTTP and ddATP. The final products were either 28 nt (unedited transcript) or 30 nt (edited transcript) in length. They were separated by 15% PAGE-urea-gel electrophoresis and visualised by autoradiography.

References

Allan GM, McNeilly F, Walker I, Linné T, Moreno-López J, Hernandez P, Kennedy S, Carroll BP, Herron B, Foster JC and Adair B (1996). A sequential study of experimental porcine paramyxovirus (LPMV) infection of pigs: immunostaining of cryostat sections and virus isolation. *J Vet Diagn Invest* **8**: 405–413.

Barrera J, Letchworth G (1996). Persistence of vesicular stomatitis virus new jersey RNA in convalescent hamsters. *Virology* **219**: 453–464.

Berg M, Sundqvist A, Moreno-López J and Linné T (1991). Identification of the porcine paramyxovirus LPMV matrix protein gene: comparative sequence analysis with other paramyxoviruses. *J Gen Virol* **72**: 1045–1050.

Berg M, Hjertner B, Moreno-López J and Linné T (1992) The P gene of the porcine paramyxovirus LPMV encodes three possible polypeptides P, V and C: the P protein mRNA is edited. *J Gen Virol* **73**: 1195–1200.

Berg M, Bergvall A, Svenda M, Sundqvist A, Moreno-López J and Linné T (1997). Analysis of the fusion protein gene of the porcine rubulavirus LPMV: analysis of paramyxovirus F protein. *Virus Genes* **14**:1, 57–63.

Hjertner BÅ, Linné T and Moreno-López J (1997). Establishment and characterisation of a porcine rubulavirus (LPMV) persistent infection in porcine kidney cells. *Acta Vet Scand* **38**: 213–224.

Hjertner BÅ, Wiman A-C, Svenda M, Berg M, Moreno-López J, Linné T (1998). Multiple factors including subgenomic RNAs and reduced viral protein expression are associated with a persistent infection by porcine rubulavirus (LPMV). *Archives of Virology* **143**: 1–15.

Letchworth G, Barrera J, Fishel I and Rodriguez L (1996). Vesicular stomatitis New Jersey virus RNA persists in cattle following convalescence. *Virology* **219**: 480–484.

Linné T, Berg M, Bergvall A, Hjertner B, Moreno-López J (1992). The molecular biology of the porcine paramyxovirus LPMV. *Veterinary Microbiology* **33**: 263–273.

Mackie EI (1981). Immunosuppressive effects of cyclophosphamide in pigs. *American Journal of Veterinary Research* **42**: 189–194.

McNeilly F, Allan GM, Moffet D, McNulty MS (1991). Detection of chicken anemia agent in chickens by immunofluorescence and immunoperoxidase staining. *Avian Pathol* **20**: 125–132.

McNeilly F, Walker I, Allan GM, Foster JC, Linné T, Merza M, Hernandez P, Kennedy S, Adair B (1997). A comparative study on the use of virus and antibody detection techniques for the diagnosis of La Piedad Michoacan Paramyxovirus (LPMV) infection in pigs. *J Vet Diagn Invest* **9**: 3–9.

Randall RE and Russel WC (1991). The paramyxoviruses In: *The paramyxoviruses*. Kingsbury DW (ed). Plenum Press: New York, pp 299–321.

Re GG (1991). Deletion mutants of paramyxoviruses. In: *The paramyxoviruses*. Kingsbury DW (ed). Plenum Press: New York, pp 275–298.

Rima B, Alexander DJ, Billeter MA, Collins PL, Kingsbury DW, Lipkind MA, Nagai Y, Örvell C, Pringle CR, ter Meulen V (1995). In: *Virus Taxonomy. Classification and nomenclature of viruses*. 6th report of the international committee on taxonomy of viruses. Murphy *et al* (eds). Archives of Virology Suppl: Springer-Verlag Wien New York: **10**: 268–74.

Sedgwick JD and Dörries R (1991). The immune system response to viral infection of the CNS. *Sem Neurosciences* **3**: pp 93–100.

Stephano AA, Gay GM and Ramirez TC (1988) Encephalomyelitis, reproductive failure and corneal opacity (blue eye) in pigs, associated with a paramyxovirus infection. *Vet Rec* **122**: 6–10.

Stephano AH (1990). Blue Eye Disease In: *Virus infections of porcines*. Pensaert MB (ed). Elsevier Science: Amsterdam, pp 237–241.

Sundqvist A, Berg M, Moreno-López J and Linné T (1992). The haemagglutinin-neuraminidase glycoprotein of the porcine paramyxovirus LPMV: comparison with other paramyxoviruses revealed the closest relationship to simian virus 5 and mumps virus. *Arch Virol* **122**: 331–340.

Svenda M, Berg M, Moreno-López J and Linné T (1997). Analysis of the large (L) protein gene of the porcine rubulavirus LPMV: identification of possible functional domains. *Virus Research* **48**: 57–70.

Svenda M, Berg M, Hjertner B, Moreno-López J and Linné T. (1998). Analysis of the nucleoprotein of porcine rubulavirus LPMV. Manuscript in preparation.

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ELSEVIER

New Form of Autosomal-Recessive Axonal Hereditary Sensory Motor Neuropathy

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Two siblings, a male and a female, had severe axonal neuropathy and sideroblastic anemia. Despite a distinct clinical picture with areflexia, ataxia, hypotonia, optic atrophy, and progressive sensory neural hearing loss, no definite diagnosis could be reached and the older sibling died at 6 years of age of respiratory failure. It is proposed that the two affected siblings have a new form of autosomal-recessive axonal hereditary sensory motor neuropathy.

Eckhardt SME, Hicks EM, Herron B, Morrison PJ, Aicardi J. New form of autosomal-recessive axonal hereditary sensory motor neuropathy. Pediatr Neurol 1998;19: 234-235.

Introduction

Two siblings, a male and a female, had severe axonal neuropathy and sideroblastic anemia. Despite a distinct clinical picture with areflexia, ataxia, hypotonia, optic atrophy, and progressive sensory neural hearing loss, no definitive diagnosis could be reached and the older sibling died at 6 years of age of respiratory failure. The diagnoses of triosephosphate-isomerase deficiency and Pearsons syndrome, as well as more common neurometabolic diseases, were excluded. It is proposed that the two affected siblings have a new form of autosomal-recessive axonal hereditary sensory motor neuropathy.

Case Report

History. The older sibling, a female, was born at term after a normal pregnancy and delivery to healthy, unrelated parents. She had four sisters all of whom are well, and there were no maternal family members with any significant medical history. She first came to medical attention because of breath-holding attacks, having initially developed normally.

When first observed at the age of 2 years, she was unsteady, with evidence of impaired vision, areflexia, and sensorineural hearing loss. She had hypotonia, ataxia, and vertical nystagmus. There was no intellectual regression. She was fully investigated, but no diagnosis was reached.

Over the next 3 years, she developed signs of a progressive neurodegenerative condition with generalized hypotonia, areflexia, and weakness affecting proximal muscle groups before distal ones and legs before arms. Later on, bulbar muscle function and dysarthria developed. Fasciculations were apparent in limbs and tongue and were then followed by peripheral sensory disturbances with cramps and paresthesia in an ascending fashion. After the initial deterioration of her vision, she developed early optic atrophy with a vertical pendular nystagmus and eventual extinction of the vestibulo-ocular reflex. In addition to the neurologic picture leading to complete wheelchair dependence and the necessity of permanent nasogastric feeding, she developed, 10 months before her death, a transfusion-dependent anemia of the sideroblastic type. Throughout her illness, her cognitive and intellectual function remained entirely intact.

Her younger brother, at 3 years of age, developed identical signs, with progressive fatigue during the past year, optic atrophy with loss of visual acuity, sensorineural hearing loss, generalized hypotonia, and ataxia. Currently, he continues to follow the same pattern of deterioration with, possibly, even faster progression. He already has neutropenia and a low normal platelet and erythrocyte count.

Test Results. The following test results were found to be normal in the older sibling: routine biochemistry, plasma amino acids, urine amino and organic acids, very long chain fatty acids, liver function tests, immunoglobulins (cerebrospinal fluid/plasma), autoimmune profile, lipid profile, trace elements, c-reactive protein, pyruvate, lactate (cerebrospinal fluid/plasma), thyroid function tests, creatine kinase, coeruloplasmin, vitamin E, A, and B_{12} levels, alpha-fetoprotein, beta-human chorionic gonadotropin, biotinidase, white cell enzymes, bile acids, bile alcohols, triose phosphate isomerase levels, and mitochondrial DNA deletion studies. Cultures of blood, cerebrospinal fluid, urine, and routine virology serology were all normal. Hemoglobin-electrophoresis, red cell folate, serum folate, cerebrospinal fluid folate, ferritin, transcobalamins (total and subclasses 1-3), Schillingtest and Frataxin gene studies, and chromosomal analysis were all normal, including chromosomal fragility studies.

She was found to have a macrocytic sideroblastic anemia with a poor reticulocyte count response. Bone marrow aspiration revealed a normal megacaryocyte and myelocyte line but marked dyserythropoesis demonstrating cells with abnormally vacuolated nuclei, ragged cytoplasm, and basophilic stippling with evidence of erythrophagocytosis.

Nerve conduction studies revealed a sensory neuropathy but without

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definite involvement of motor neurones. The sural nerve biopsy revealed a chronic axonal neuronopathy affecting myelinated and unmyelinated fibers with some loss of the latter but no regeneration. Muscle biopsy demonstrated morphologically normal muscle and no evidence of denervation. There were no ragged red fibers and no evidence of any metabolic disturbance (normal lipid content, cytochrome oxidase, and reduced form of nicotinamide-adenine dinucleotide dehydrogenase activity). Electron microscopy did not reveal abnormal mitochondria.

The electroretinogram was normal, but the visual-evoked responses were markedly attenuated and degraded, indicating severe postretinal dysfunction with poor acuity levels. Repeat examination demonstrated a progressive loss of the vestibulo-ocular reflex with almost complete absence, indicating brainstem disease or a peripheral neuropathy with possible demyelination. The associated eye movement disorder could not be classified.

Repeated imaging of the brain and spinal cord by computed tomography and magnetic resonance imaging at 24 months and 6 months before her death failed to reveal any abnormality; in particular, the brainstem and cerebellum appeared normal.

A full postmortem examination was performed. The pathologic findings were predominantly in the nervous system. The cause of death was bronchopneumonia. No pathologic finding was detected in the pancreas or spleen. On examination of the nervous system the cortical neuronal population appeared reduced, and there was an increase in white matter neurons. There was no hypoxic-ischemic necrosis and no hyppocampal sclerosis. The deep white matter demonstrated degeneration in the projection fibers extending from the cortex to the brainstem, most likely secondary to the neuronal loss. The cerebellum had marked abnormality with cell loss and gliosis in the dentate nucleus and wallerian degeneration in associated white matter tracts in the cerebellar peduncles. Symmetric foci of neuronal loss, neuronophagia, and gliosis were present throughout the brainstem, affecting cranial nerve nuclei. The spinal cord was examined at multiple levels. There was severe loss of anterior horn cells with neuronophagia and gliosis. Long tract degeneration was present, affecting spinocerebellar, spinothalamic, posterior columns, and corticospinal tracts, and posterior columns at all levels of the cord.

Eye examination revealed severe degenerative changes in the peripheral retina but the central retina appeared intact. The optic nerve and optic tract had marked myelin pallor, and there was cell loss and gliosis in the lateral geniculate body.

Postmortem examination of muscle from biceps, quadriceps, and tongue revealed neurogenic atrophy. Peripheral motor and sensory nerves demonstrated a chronic axonal neuropathy affecting myelinated and unmyelinated fibers with no evidence of regeneration.

Discussion

There were two stages of clinical deterioration. Initially the patient presented with ataxia and visual and hearing loss. Later there was progressive anterior horn cell and bulbar dysfunction. Cognitive decline was not a feature throughout her illness.

The clinical signs of ataxia, visual impairment with optic atrophy, nerve VIII dysfunction, and bulbar dysfunction point to a possibly demyelinating brainstem disease. However, neither imaging nor white cell enzyme function studies could confirm that or a primary white matter disorder at any stage of her disease.

The postmortem examination demonstrated a multisystem disorder affecting the visual system, cerebellum motor and sensory systems, brainstem, and spinal cord.

The peripheral sensory neuropathy with abnormal nerve conduction and the nerve biopsy findings could not be classified into any known hereditary sensory motor neuropathy. However, an autosomal-recessive spinocerebellar ataxia with optic atrophy and deafness has been described [1].

Although there is no known link between the hereditary sensory-motor neuropathies and the pathologic bone marrow findings, linkage between the Duffy locus, HLA antigens, and a hereditary sensory-motor neuropathy has been found [2].

The transfusion-dependent anemia that developed late in the disease is significant. The presence of normal exo- and endocrine pancreatic function and histologic findings, normal lactate measurements (plasma and cerebrospinal fluid), and no detectable mitochondrial deletion (including mutation T8993G) or mutation excludes the diagnosis of Pearson syndrome.

Although a diagnosis of a mitochondrial disorder could not be made, our case with the atypical retinitis, anemia, hypotonia, and ataxia resembles some features of a previously described case of a patient surviving Pearsons syndrome and developing Kearns-Sayre syndrome [3].

This case most closely resembles the picture of triosephosphate-isomerase deficiency [4], but normal enzyme level assays in both siblings excluded the diagnosis.

A hereditary sensory neuropathy with progressive destruction of neural and bone marrow precursor cells is hence considered likely. A mitochondrial disorder and any of the known hereditary sensory motor neuropathies were excluded, as was a primary demyelinating disease.

Because the condition seemed to create abnormalities outside the nervous system (bone marrow), an underlying metabolic condition is likely to be the cause of this new condition, which cannot yet be diagnosed biochemically.

The authors suggest that these patients represent a new neurometabolic disorder that is likely to be autosomal recessive in inheritance. Further research involving the genetics of the hematologic antigen system and its possible link to the pathologic destructive process will be required. In view of the possibility of this disease representing a new mitochondrial disorder, further gene sequencing of mitochondrial DNA in these cases is required.

References

[1] **Nikali K**, Iosumppi J, Lonnquist T, Mao J, Suomalainen A, Peltoen L. Towards cloning of a novel ataxia gene: Refined assignment and physical map of the IOSCA locus (SCA8) on 10q24. Genomics 1997;39:185-91.

[2] **Nevsimalova S**, Prazal J, Herzog P, Seemanova E. Duffy locus linkage and HLA antigens in hereditary motor-sensory neuropathy. Schweiz Arch Neurol Psychiatr 1991;142:19-29.

[3] **Somonsz HJ**, Barlocher K, Rotig A. Kearns-Sayre syndrome developing in a boy who survived Pearsons syndrome caused by a mitochondrial DNA deletion. Doc Ophthalmol 1992;82:73-9.

[4] **The BT Poll**, Aicardi J, Girot R, Rosa R. Neurological findings in triosephosphate isomerase deficiency. Ann Neurol 1985;17:117-24.

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Apoptosis in measles virus-infected human central nervous system tissues

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Apoptosis in measles virus-infected human central nervous system tissues

The extent of apoptotic cell death was examined in central nervous system (CNS) tissues from three cases of subacute sclerosing panencephalitis (SSPE). Apoptosis was demonstrated by *in situ* end-labelling of DNA in formalin-fixed, paraffin-embedded tissue sections. Measles virus and cell types were labelled by immunohistochemistry and/or *in situ* hybridization. Furthermore, bcl-2 expression in SSPE was examined by immunohistochemistry. All three cases exhibited varying degrees of apoptosis in all CNS areas studied. Brain tissue from a non-neurological control case did not show any significant apoptosis. Characterization of cell types demonstrated neurons, oligodendrocytes, lymphocytes and microglia undergoing apoptosis. A linear relationship could not be established between virus burden and the extent of apoptosis in any particular area. Virus-negative cells were observed which were undergoing apoptosis. Bcl-2 immunoreactivity in SSPE was confined to the infiltrating cell population. These results suggest that apoptosis of various cell types may contribute to the neuropathogenesis of measles virus infection in the human CNS, either as a direct effect of viral infection or by cytokine-mediated responses.

Keywords: measles virus, CNS, apoptosis, *in situ* end-labelling, bcl-2

Introduction

Apoptosis is a physiological form of cell death characterized by deletion of individual cells; it differs from necrosis both morphologically and biochemically. It is a natural process occurring during development and morphogenesis, in the normal turnover of tissues, in immune system maturation and in response to suppression of external signals from other cells (e.g. growth factor withdrawal). Apoptosis also occurs in various pathological conditions, most notably in tumours in which it is responsible for continuous cell loss [9]. Furthermore, inhibition of apoptosis by expression of the proto-oncogene bcl-2 is thought to be one of the mechanisms involved in the development of tumours [17]. The bcl-2 protein is a critical regulator of programmed cell death and has been implicated as playing a role in the selection of primed/memory T-cells during acute viral infections [2, 3]. *In vitro*, bcl-2 has been proven capable of protecting neurons from apoptosis [6].

Recently, a number of viruses have been shown to cause cell death by induction of apoptosis [14, 18, 24, 31]. Conversely, viruses that can cause persistent infection may develop mechanisms that prevent or delay the induction of apoptosis or may persist in cell types resistant to virus-induced cell death [15]. In the central nervous system (CNS), apoptosis has been shown to be a mechanism for neuronal cell death in productive infection with human immunodeficiency virus [1, 12].

Subacute sclerosing panencephalitis (SSPE), is a rare complication of measles virus (MV) infection of the CNS which generally occurs many years after the initial infection. The pathological features of MV in the CNS include widespread neuronal and oligodendrocytic infection, perivascular inflammation, neuronal loss, gliosis and demyelination [5]. While apoptosis has been

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observed in MV infection of cells in culture [10], the role or extent of apoptosis in the pathogenesis of SSPE has not been described.

The development of the *in situ* end-labelling (ISEL) technique has facilitated detection of apoptotic cells in routinely fixed biopsy and post-mortem tissues [11, 13, 20, 34]. This method is based on the recognition of 3'-OH ends of DNA by terminal deoxynucleotide transferase that incorporates hapten-labelled oligonucleotides at the sites of the DNA strand breaks which occur during apoptosis.

Using immunocytochemistry and *in situ* hybridization for detection of MV and ISEL for marking cells undergoing apoptosis, the relationship between virus burden and apoptosis in MV-infected human CNS tissue was examined. Cell-specific markers were used to identify the cell types undergoing apoptosis. In addition, the expression of bcl-2 in the same brain areas of SSPE was examined.

Materials and methods

Tissue

Three autopsy cases of SSPE were included on the basis of clinical features, cerebrospinal fluid and serum measles IgG antibody titres and pathological examination [5]. SSPE tissues were fixed in 10% formalin for 2 weeks before routine processing to paraffin. Blocks were selected from the frontal and temporal cortices, cerebellum, medulla and spinal cord from each case. Blocks from the same areas of a normal brain were fixed and processed in a similar fashion as controls. Blocks of normal tonsil were used as controls for bcl–2. Sections were cut from all blocks for histology, immunocytochemistry, *in situ* hybridization and ISEL.

Antibodies and immunocytochemistry

The following mouse monoclonal (mAb) and polyclonal antisera were used: polyclonal antiserum to MV (1:2000, Dr Mark Gödec, National Institute for Health, Bethesda, Maryland, USA); mAb to human bcl-2 protein (1:50, Dako); mAb to GFAP (1:100, Dako); mAb to CD68 (1:50, Dako); mAb to LCA (1:50, Dako); and mAb to CD34 (1:20, Serotec, England). All of the cell-specific markers are known to be specific for the cell types described [4, 21]. For exposure of viral antigens, GFAP, CD68, CD34 and bcl-2 antigens in SSPE and normal brain tissue, a microwave antigen retrieval system was employed as described previously [21]. All primary antibodies were incubated overnight at 4 °C and bound antibodies were detected as described previously [21].

In situ hybridization

A biotinylated single-stranded RNA probe to the nucleocapsid gene of MV was prepared by subcloning the nucleocapsid gene sequence into a gemini *in vitro* transcription vector [8]. *In situ* hybridization and immunodetection of the biotinylated hybrids was carried out as described elsewhere [22].

In situ tailing

Fragmented DNA was detected in paraffin-embedded tissue sections using an ISEL method [13]. Briefly, sections were dewaxed overnight in xylene and rehydrated to water through graded ethanols. Following 2 × 5 min washes in 50 mM Tris buffered saline pH 7.6 (TBS) sections were incubated with proteinase K (Sigma) at room temperature (0.1–0.5 mg/ml in TBS 5–20 min). Excessive digestion with proteinase K led to staining of morphologically normal nuclei both in the SSPE and normal tissues. Optimal staining reaction was observed when sections were incubated in 0.5 mg/ml proteinase K for 15 min.

All sections were then washed 2 × 5 min in TBS. Sections were then incubated in an ISEL mix for 1 h at 37 °C. This consisted of 10 µl 5x tailing buffer, 1 µl DIG-dNTP (Boehringer 1277065), 2 µl $CoCl_2$, 0.5 µl terminal transferase (Boehringer 220582) and 36.5 µl sterile, distilled H_2O. Detection of incorporated digoxigenin-labelled nucleotides was carried out as described previously using anti-digoxigenin-alkaline phosphatase and Fast Blue BB salt [13]. Subsequently, sections were lightly counterstained in Nuclear Fast Red and mounted in glycerine jelly. Terminal transferase was omitted from the ISEL mixture as a negative control.

Double-labelling ISEL/immunocytochemistry

To investigate further the relationship between apoptosis and virus infection of specific cell types, selected sections were double-labelled for apoptosis and either MV antigen

Table 1. Semi-quantitative analysis of the relationship between measles virus infection and apoptosis in three cases of SSPE

Case No. (age/disease duration)	Brain area									
	Frontal		Temporal		Cerebellum		Medulla		Spinal cord	
	MV*	ISEL†	MV*	ISEL†	MV*	ISEL†	MV*	ISEL†	MV*	ISEL†
A (22 y/2 months)	3	3	3	3	0	1	2	2	0	2
B (20 y/14 months)	2	2	3	3	0	1	1	2	0	ND
C (10 y/4 months)	3	1	3	1	1	0	1	1	0	1
Normal brain	0	0	0	0	0	0	0	0	0	0

*Number of measles virus-positive cells determined by immunocytochemistry or *in situ* hydridization. 0, None. 1, Few. 2, Moderate. 3, Abundant. †Extent of apoptosis determined by *in situ* end-labelling. 0, No apoptosis. 1, Few apoptotic cells. 2, Moderate numbers of apoptotic cells. 3, Scattered apoptosis throughout tissue block. ND, Not done.

or cell-specific markers. ISEL was carried out on the sections and after washing in running tap water sections were reacted with 5% normal rabbit or swine serum then incubated overnight at 4 °C in measles virus, GFAP, CD68, CD34 or LCA antibodies. Reaction sites were developed as described for single-labelling. No counterstain was applied to the sections which were mounted in glycerine jelly.

Immunocytochemistry, *in situ* hybridization and ISEL results were examined and scored independently by at least two observers. A semi-quantitative scoring system was used to determine burden of virus and numbers of apoptotic cells in various anatomical areas (see Table 1).

Results

Virus detection

Measles virus was demonstrated by both immunocytochemistry for viral antigen and by *in situ* hybridization for viral genomic RNA in all three SSPE cases (Table 1). Similar numbers of cells were labelled by both techniques in all blocks. Neurons and oligodendrocytes were the cell types most frequently positive. Occasionally MV-positive cerebral astrocytes, endothelial, interparenchymal macrophages and perivascular inflammatory cells were observed. This was not, however, a prominent feature in any of the cases and was confined to areas of heavy infection. All blocks from the normal control case were negative for viral antigen or RNA.

Detection of apoptotic cells in SSPE tissue

All three SSPE cases exhibited cells undergoing apoptosis in all blocks studied, but to varying extents (Table 1). Many cells showing histological criteria of apoptosis were observed by haematoxylin and eosin staining but these were much fewer in number than those observed by ISEL. In areas of tissue with few apoptotic cells these were confined to either the perivascular cuffs or very occasionally in the tissue parenchyma. In tissue areas, where scattered apoptosis was observed throughout the tissue section, both grey and white matter cells (Figure 1a) were labelled.

As indicated in Table 1, no clearly identifiable relationship was established between the numbers of cells undergoing apoptosis and the extent of viral infection. Furthermore, no correlation was observed between the degree of lymphocytic infiltration and the extent of apoptosis in any brain area. However, careful examination of serial sections and of sections double-labelled for apoptosis and MV antigen revealed both virus-positive (Figure 1b), and virus-negative cells (Figure 1c), which were undergoing apoptosis. These were identified in both grey and white matter. In case A moderate numbers of cells undergoing apoptosis were observed in the cervical spinal cord in the absence of demonstrable virus. Conversely, in case C it was noted that the extent of viral infection in the frontal and temporal lobes was much greater than the number of cells undergoing apoptosis. However, there was no difference in the cellular distribution of virus in this case compared with cases A and B.

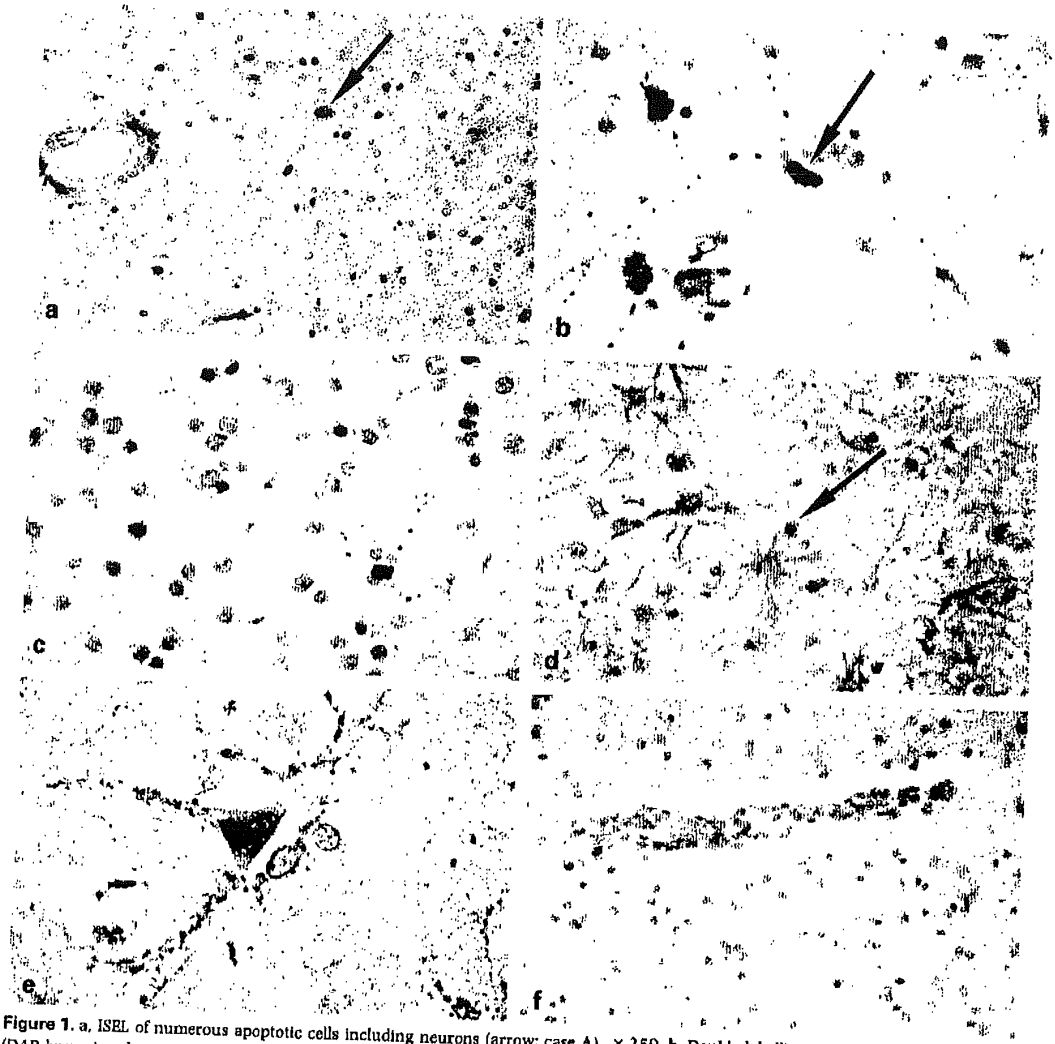

Figure 1. a, ISEL of numerous apoptotic cells including neurons (arrow; case A), × 250. b, Double-labelling for MV antigen (DAB-brown) and apoptosis (blue), one cell shows both reaction products (arrow). c, Double-labelling showing cells that do not contain viral antigen undergoing apoptosis (b, c both case C), × 400. d, Double-labelling for GFAP (DAB-brown) and apoptosis (blue). The GFAP-positive astrocyte is not apoptotic but the cell with oligodendrocytic morphology is apoptotic (arrow; case A), × 400. e, Double-labelling for CD68 (DAB-brown) and ISEL shows a neuron undergoing apoptosis (case A), × 1000. f, SSPE brain tissue demonstrating many bcl-2-positive cells in a perivascular cuff, × 250.

Identification of cell types undergoing apoptosis

Cell types undergoing apoptosis were identified either morphologically or by sequential labelling of ISEL-stained sections with cell-specific markers. ISEL revealed neurons undergoing apoptosis in all three SSPE cases (Figure 1a,e). Positive reaction was located either over the whole nucleus or in the periphery of the nucleus. Some neurons were observed to have reached the end stage of apoptosis and were about to be engulfed by microglia. Infiltrating lymphocytes (detected by LCA)

were ISEL-positive both in the perivascular cuffs and in the brain parenchyma. Positive staining of process bearing cells, which were labelled by the macrophage-specific marker CD68, was also observed throughout the CNS in all three cases of SSPE. Astrocytes, as identified by GFAP staining, were not observed undergoing apoptosis. However, many cells with the morphological appearance of oligodendrocytes were observed undergoing apoptosis (Figure 1b). Apoptosis was also detected in cerebral endothelial cells (characterized by CD34 immunoreactivity).

Controls

No labelled cells were observed when terminal transferase was omitted from the ISEL reaction mix. Sections from the blocks from the normal control brain showed very few cells undergoing apoptosis. These were identified in the white matter and were identified by morphology as oligodendrocytes or resting microglia.

Bcl-2 protein expression

Tonsil tissue

The most intense immunostaining was observed in the follicular mantle. Bcl-2 immunoreactivity within cells was observed as a granular, punctate cytosolic pattern. Lymphocytes within the interfollicular regions were also often positive. However, the majority of cells within the germinal centre were bcl-2 negative.

SSPE tissue

Intense immunostaining was seen in the cells of the perivascular cuffs (Figure 1f), and in occasional infiltrating cells in the white matter parenchyma. However, neurons and glia cells were consistently negative in all three cases. No bcl-2 immunoreactivity was seen in the tissue blocks from the normal case.

Discussion

In the present study ISEL was used on paraffin sections to demonstrate that apoptotic cells are widespread in MV infection of the human CNS. A detailed description of the extent of virus distribution in SSPE is given in a recent publication from this laboratory [5].

A previous cell culture study by Esolen *et al.* of Vero cells and monocytic cell lines has shown that MV infection was capable of inducing apoptosis [10]. Furthermore, they showed that the nuclei of cells recruited into syncytia are initially normal and then develop evidence of endonucleolytic cleavage of chromosomal DNA. The present study extends these observations *in vivo* and demonstrates that neuronal cells may also be induced to undergo apoptosis as a result of MV infection of the CNS.

Apoptosis was demonstrated in a wide range of cell types in the CNS in SSPE. Neurons in the grey matter, endothelial cells and cells in the perivascular cuffs within blood vessel walls and infiltrating lymphocytes were all observed undergoing apoptosis, albeit in differing numbers. Apoptosis of cells with the morphological characteristics of oligodendrocytes and microglia was also observed.

Numerous studies have shown evidence for neuronal apoptosis [7, 19, 33]. However, there are few reports of apoptosis in neurons in virus infections of the CNS. Apoptosis of neurons has been observed in human immunodeficiency virus-type 1 (HIV)-associated dementia in adults [1, 12], where it is suggested to be a delayed phenomenon secondary to microglial activation. The present study represents the first report of apoptosis in neurons in MV infection of the CNS.

Lymphocyte and macrophage apoptosis has been demonstrated in many situations in the CNS [12, 26, 27, 29]. For example, Schmied *et al.* demonstrated that a high percentage of T lymphocytes in experimental autoimmune encephalomyelitis showed signs of apoptosis at the time of recovery from disease. They suggested that apoptosis of T lymphocytes may be one possible mechanism to eliminate T lymphocytes from inflammatory brain lesions. Lymphocytes undergoing apoptosis in SSPE may be subject to similar elimination mechanisms. Alternatively, direct virus infection could lead to cell death by apoptosis of individual lymphocytes.

Double-labelling revealed that the number of apoptotic cells was sometimes greater than those containing detectable levels of virus. However, it is possible that very low levels of virus (and therefore undetectable by conventional immunocytochemistry or *in situ* hybridization) may still induce apoptosis. Alternatively, non-infected cells may be induced to undergo apoptosis indirectly, possibly by the action of cytokines.

In both SSPE and HIV-associated dementia, activated microglia, lymphocytes or reactive astrocytes (infected and/or uninfected) could release cytokines likely to mediate neurotoxicity. Candidate cytokines include Il-2, TNFβ and TNFα [25, 30, 32]. The finding of apoptosis in apparently uninfected cells in diseases such as SSPE and HIV dementia may be important in the context of CNS disorders of unknown aetiology. For example in multiple sclerosis, primary viral infection could, through cytokine release, trigger secondary effects such as apoptosis in oligodendrocytes, leading to demyelination.

Our findings of strong immunoreactivity to bcl-2 in lymphocytes in SSPE is expected due to the overexpression of bcl-2 in activated T- and B-cells [16, 28]. The result of such overexpression of bcl-2 is to protect these cells from apoptosis. Immunoreactivity to bcl-2 was not observed in neurons or glial cells in SSPE or normal brain. There are conflicting reports in the literature on bcl-2 expression in human neurons. Merry *et al.* concluded that in the majority of post-mitotic CNS neurons bcl-2 levels are greatly reduced or immunocytochemically undetectable [23]. Other studies have demonstrated bcl-2 activity in neurons [16, 33]. However, the discrepancy in results may reflect the different fixation regimes involved. Hockenbery *et al.* used brain tissue processed as frozen sections to demonstrate bcl-2 staining in the dendritic cytoplasm of neurons while Troost *et al.* used formalin-fixed tissues but did not detail the length of fixation. It is possible that due to the length of formalin fixation in the cases used in the present study (4–6 weeks), that bcl-2 expression in neurons is below the threshold of detection. The use of snap-frozen tissue from cases of SSPE, which was unavailable for this study, would be required to assess bcl-2 restriction in neurons in response to virus infection.

In conclusion, apoptosis is a constant finding in MV infection in the CNS. However, it does not correlate with the apparent degree of viral infection or with the extent of lymphocytic infiltration at any site. The role of apoptosis in contributing to the underlying pathology of the disease process will be the subject of further investigations.

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References

1 Adle-Biassette H, Levy Y, Colombel M, Poron F, Natchev S, Keohane C, Gray F. Neuronal apoptosis in HIV infection in adults. *Neuropathol Appl Neurobiol* 1995; 21: 218–27

2 Akbar AN, Borthwick N, Salmon M *et al.* The significance of low bcl-2 expression by CD45RO T cells in normal individuals and patients with acute viral infections. The role of apoptosis in T cell memory. *J Expl Med* 1993; 178: 427–38

3 Akbar AN, Salmon M, Janossy G. Role of bcl-2 and apoptosis in viral infections. *Int Arch Allergy Immunol* 1994; 105: 359–62

4 Allen IV, McQuaid S, McMahon J, Crangle K, McConnell R. The expression of the endothelial cell antigen CD34 in demyelinating disease. *Neuropathol Appl Neurobiol* 1996; 22: 101–7

5 Allen IV, McQuaid S, McMahon J, Kirk J, McConnell R. The significance of measles virus antigen and genome distribution in the CNS in SSPE for mechanisms of viral spread and demyelination. *J Neuropathol Exp Neurol* 1996; 55: 471–80

6 Allsopp TE, Wyatt S, Paterson HF, Davies AM. The proto-oncogene bcl-2 can selectively rescue neurotrophic factor dependent neurons from apoptosis. *Cell* 1993; 73: 295–307

7 Brück Y, Brück W, Kretzschmar HA, Lassmann H. Evidence for neuronal apoptosis in pontosubicular neuron necrosis. *Neuropathol Appl Neurobiol* 1996; 22: 23–9

8 Cosby SL, McQuaid S, Taylor MJ, Bailey M, Rima BK, Martin SJ, Allen IV. Examination of eight cases of multiple sclerosis and 56 neurological and non-neurological controls for genomic sequences of measles virus, canine distemper virus, simian virus 5 and rubella virus. *J Gen Virol* 1989; 70: 2027–36

9 Cotter TG, Lennon SV, Glynn JG, Martin SJ. Cell death via apoptosis and its relationship to growth, development and differentiation of both tumour and normal cells. *Anticancer Res* 1990; 10: 1153–60

10 Esolen LM, Park SW, Hardwick JM, Griffin DE. Apoptosis as a cause of death in measles virus-infected cells. *J Virol* 1995; 69: 3955–58

11 Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death *in situ* via specific labelling of nuclear DNA fragmentation. *J Cell Biol* 1992; 119: 493–501

12 Gelbard HA, James HJ, Sharer LR *et al.* Apoptotic neurons in brains from paediatric patients with HIV-1 encephalitis and progressive encephalopathy. *Neuropathol Appl Neurobiol* 1995; 21: 208–17

13 Gold R, Schmeld M, Rothe G, Zischler H, Breitschopf H, Wekerle H, Lassmann H. Detection of DNA fragmentation in apoptosis: application of *in situ* nick translation to cell culture systems and tissue sections. *J Histochem Cytochem* 1993; 41: 1023–30

14 Gougeon M, Garcia S, Heeney J *et al.* Programmed cell death in AIDS-related HIV and SIV infections. *AIDS Res Hum Retroviruses* 1993; 9: 553–63

15 Henderson S, Huen D, Rowe M, Dawson C, Johnson GI, Rickinson A. Epstein-Barr virus-coded BHRF1 protein, a viral homologue of bcl-2, protects human B cells from programmed cell death. *Proc Natl Acad Sci USA* 1993; 90: 8479–83

16 Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ. Bcl-2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc Natl Acad Sci USA* 1992; 88: 6961–5

17 Ikeda H, Hirato J, Akami M, Matsuyama S, Suzuki N, Takahashi A, Kuroiwa M. Bcl-2 oncoprotein expression and apoptosis in neuroblastoma. *J Pediatr Surg* 1995; 30: 805–8

18 Jeurissen SHM, Wagenaar F, Pol JMA, van der Eb AJ, Noteborn MHM. Chicken anaemia virus causes apoptosis of thymocytes after *in vivo* infection and of cell lines after *in vitro* infection. *J Virol* 1992; 66: 7383–88

19 Kihara S-I, Shiraishi T, Nakagawa S, Toda K, Tabuchi K. Visualization of DNA double strand breaks in the gerbil hippocampal CA1 following transient ischemia. *Neurosci Lett* 1994; 175: 133–6

20 Lucassen PJ, Wilson CJC, Vermeulen JP, Van Lookeren-Campagne M, Van Dierendonck JH, Swaab DF. Microwave-enhanced *in situ* end-labelling of fragmented DNA: parametric studies in relation to postmortem delay and fixation of rat and human brain. *J Histochem Cytochem* 1995; 43: 1163–71

21 McQuaid S, McConnell R, McMahon J, Herron B. Microwave antigen retrieval for immunocytochemistry on formalin-fixed, paraffin-embedded post-mortem CNS tissue. *J Pathol* 1995; 176: 207–16

22 McQuaid S, Allan GM. Detection protocols for biotinylated probes: optimization using multistep techniques. *J Histochem Cytochem* 1992; 40: 569–74

23 Merry DE, Veis DJ, Hickey F, Korsmeyer SJ. Bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. *Development* 1994; 120: 301–11

24 Meyaard L, Otto SA, Jonker RR, Mijnster MJ, Keet RPM, Miedema F. Programmed death of T cells in HIV-1 infection. *Science* 1992; 257: 217–19

25 Nagano I, Nakamura S, Yoshioka M, Onodera J, Kogure K, Itoyama Y. Expression of cytokines in brain lesions in subacute sclerosing panencephalitis. *Neurology* 1994; 44: 710–15

26 Nguyen KB, McCombe PA, Pender MP. Macrophage apoptosis in the central nervous system in experimental autoimmune encephalomyelitis. *J Autoimmunity* 1994; 7: 145–52

27 Pender MB, Nguyen KB, McCombe PA, Kerr JFR. Apoptosis in the nervous system in experimental autoimmune encephalomyelitis. *J Neurol Sci* 1991; 104: 81–7

28 Pezzella F, Tse AGD, Cordell JL, Pulford KAF, Gatter KC, Mason DY. Expression of the bcl-2 oncogene protein is not specific for the; 14: 18 chromosomal translocation. *Am J Path* 1990; 137: 225–32

29 Schmeid M, Breitschopf H, Gold R, Zischler H, Rothe G, Wekerle H, Lassmann H. Apoptosis of T lymphocytes in experimental autoimmune encephalomyelitis. Evidence for programmed cell death as a mechanism to control inflammation in the brain. *Am J Path* 1993; 143: 446–52

30 Schneider-Schaulies J, Schneider-Schaulies S, Ter Meulen V. Differential induction of cytokines by primary and persistent measles virus infection of human glial cells. *Virology* 1993; 195: 219–28

31 Takizawa T, Matsukawa S, Higuchi Y, Nakamura S, Nakanishi Y, Fukuda R. Induction of programmed cell death (apoptosis) by influenza virus infection in tissue culture cells. *J Gen Virol* 1993; 74: 2347–55

32 Talley A, Perry S, Epstein LG, Gelbard HA. TNFα-mediated apoptosis in SK-N-MC neuroblastoma cells: a model for neuronal cell loss in HIV-1 associated dementia. *Ann Neurol* 1994; 36: 506–7

33 Troost D, Aten J, Morsink F, de Jong JMBV. Apoptosis in amyotrophic lateral sclerosis is not restricted to motor neurons. Bcl-2 expression is increased in unaffected post-central gyrus. *Neuropathol Appl Neurobiol* 1995; 21: 498–504

34 Wijsman JH, Jonker RR, Keijzer R, Van de Velde CJH, Cornelisse CJ, Van Dierendonck JH. A new method to detect apoptosis in paraffin sections: *in situ* end-labelling of fragmented DNA. *J Histochem Cytochem* 1993; 41: 7–12

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A Sequential Study of Experimental Porcine Paramyxovirus (LPMV) Infection of Pigs: Immunostaining of Cryostat Sections and Virus Isolation

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A sequential study of experimental porcine paramyxovirus (LPMV) infection of pigs: immunostaining of cryostat sections and virus isolation

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Abstract. La Piedad Michoacan Paramyxovirus (LPMV) is a recently recognized paramyxovirus infecting pigs throughout Mexico. Disease syndromes observed in field cases associated with LPMV infection include neurologic, respiratory, and reproductive disorders. Clinical signs and the distribution of LPMV virus and antigen in tissue samples from pigs experimentally infected with LPMV by natural routes were studied. Severe neurologic disease and death occurred following experimental inoculation of 3- and 17-day-old pigs. All of the pigs inoculated at 3 days of age were either dead or moribund by 8 days after inoculation, whereas 30% of the pigs inoculated at 17 days of age were affected. Virus was consistently recovered from or demonstrated in tissues from the respiratory tract of both groups of pigs. LPMV and antigen were also demonstrated in central nervous system (CNS) tissues from these pigs; however, differences in virus distribution within the CNS were demonstrated in the 2 groups. In the pigs inoculated at 17 days of age, isolation of LPMV was restricted to the olfactory bulb and midbrain. In contrast, in the pigs inoculated at 3 days of age, isolation of LPMV was more widespread throughout the CNS tissue examined. Virus excretion studies indicated that nasal spread of LPMV was more important than fecal spread. Comparatively large quantities of infectious LPMV were consistently recovered from urine samples of experimentally infected pigs.

An outbreak of a fatal disease of pigs in Mexico (El Sindrome del Ojo Azul), associated with an unrecognized infectious agent, was first reported in 1981.[10] This outbreak of disease was characterized by encephalomyelitis and corneal opacity (blue eye) in young pigs and reproductive disorders in adult pigs. The clinical signs observed in this outbreak of disease were variable and dependant on the age of the pigs infected. Typically, the disease in piglets 2-15 days of age usually started with fever, a rough hair coat, and an arched back. These signs were quickly followed by progressive nervous signs of ataxia, weakness, rigidity, muscle tremor, abnormal posture, and death within 48 hours. In older pigs, clinical signs were much less severe, consisting of transient fever, sneezing, and coughing. Nervous system signs were less common and less obvious, and mortality rates were very low. The causal agent was confirmed as a paramyxovirus and named La Piedad Michoacan paramyxovirus (LPMV)[7] or blue eye paramyxovirus.[8] Since the initial report, LPMV infection of pigs has spread throughout Mexico with disease outbreaks recorded in many states.[9] Portions of the genome of LPMV have recently been sequenced, and some homology between LPMV and the mumps virus group of paramyxoviruses has been reported.[2,4,12] Although clinical signs, histopathology, and tissue distribution of virus following naturally occurring LPMV infection of pigs have been reported,[9] there are few detailed studies of the pathogenesis of the porcine paramyxovirus following experimental infection by natural routes.[9] Here, we report the results of a detailed sequential study of virus distribution in tissues of pigs experimentally infected with LPMV by a natural route, as determined by immunofluorescence staining and virus isolation techniques. In addition, we report the results of virus excretion studies following experimental infection of pigs with LPMV.

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Materials and methods

Virus. A cell culture isolate of the porcine paramyxovirus, designated LPMV-85, was used throughout this study. This

virus was originally isolated in Mexico from brain tissue of a pig exhibiting clinical signs typical of LPMV infection[10] and has been characterized at protein and genomic levels.[1,2,4,11,12] Following receipt of this isolate at the Veterinary Sciences Division, the virus was purified by 3 passages at limiting dilution in continuous pig kidney cell cultures (PK/15), and a working pool was prepared. The infectious titer of this working pool was calculated to be $10^{7.50}$ $TCID_{50}$/0.1 ml, following inoculation of serial 10-fold dilutions in PK/15 cell cultures. This virus pool was examined for evidence of contamination with pseudorabies virus, classical swine fever virus, and hemagglutinating encephalitis virus using direct immunofluorescence (IF) staining of acetone-fixed cell culture preparations. No evidence of contamination of the LPMV preparation with these viruses was detected.

Antiserum. A hyperimmune antiserum to the LPMV-85 isolate was prepared in a rabbit. Initially, this rabbit was inoculated with 1 ml of the virus working pool mixed with an equal volume of purified saponin[a] injected intramuscularly at 4 sites. Four weeks later, the rabbit was given an intravenous inoculation of 0.5 ml of the LPMV-85 virus alone, and blood samples were collected after a further 2 wk. The immunoglobulin from this sample was separated and conjugated to fluorescein isothiocyanate (FITC).[5] This conjugate was then titrated by serial 2-fold dilution in phosphate buffered saline (PBS) (pH 7.2) and applied to acetone-fixed coverslip preparations of LPMV-infected PK/15 cell cultures. The IF titer was 1/10,024, and the conjugate was used at a 1/400 dilution in PBS for immunostaining of acetone-fixed cell cultures and cryostat sections of tissues.[5]

Pigs. All the pigs used in this study were obtained from a closed, minimal disease Large White breeder/finisher unit. These animals were shown to be free of infection with LPMV, pseudorabies virus, porcine parvovirus, and classical swine fever virus by using indirect IF tests on sera from these pigs on acetone-fixed virus-infected cell cultures.

Experiment 1. Thirty 3-day-old colostrum-fed pigs were used in this experiment. These animals were obtained from 3 litters, housed in animal accommodation under negative air pressure and fed a mixture of equal volumes of 1% dextrose in water and full cream evaporated milk[b] for the duration of the experiment. Twenty-seven pigs were each inoculated by intranasal and eyedrop routes with $10^{7.00}$ $TCID_{50}$ of the LPMV-85 virus pool. This inoculum was administered in 2 5-ml doses 6 hr apart. One pig was euthanized on each of days 1, 2, 3, 4, 5, 7, and 8 after infection by intravenous barbiturate injection, and tissue samples (Table 1) were processed for virus isolation and cryostat sectioning. For virus isolation, approximately 10% (w/v) suspensions of each of the tissue samples were prepared in minimal essential medium containing Earle's salts and 100 µg/ml of gentamicin. Following centrifugation at 3,000 x g for 30 min, the supernatant fluids were removed and inoculated into PK/15 cell culture preparations, and the cultures were incubated for 6 days at 37 C. The cell cultures were then subjected to 1 freeze/thaw cycle, and the resulting cell lysate was assayed for hemagglutination (HA) activity using 0.8% chicken red blood cells in PBS. HA-positive lysates were inoculated into coverslip cell culture preparations of PK/15 cells, and after incubation at 37 C for 24 hr. the coverslip preparations were fixed in acetone and immunostained for LPMV antigen using the FITC conjugate. HA-negative cell lysates were inoculated into fresh PK/15 cell cultures, incubated for a further 6 days at 37 C, and assayed for HA and IF. Tissue samples for cryostat sectioning were processed and immunostained.[5]

Three pigs held in separate animal accommodation were used as controls. These animals were inoculated with 10 ml of uninfected PK/15 cell lysate using the procedures described above, and 1 pig was euthanized on each of days 1, 4, and 8 after inoculation. Tissue samples for virus isolation and immunostaining were taken from these animals and processed as described above.

Experiment 2. Thirty 17-day-old pigs were used in this experiment. These animals were housed in accommodation similar to that described above for the 3-day-old pigs. Twenty-seven pigs in this group were inoculated with LPMV using the same virus pool, volume of inoculum, and inoculation schedule as for the 3-day-old pigs. One pig from this group was euthanized on each of days 1, 2, 3, 4, 5, 7, 8, 9, 11, and 14 after inoculation, and tissue samples were taken (Table 2) and processed for virus isolation and immunostaining of cryostat sections as described above.

Three pigs from this group held in separate accommodation were used as controls. These animals were inoculated with uninfected PK/15 cell lysates, and tissues were processed as described for the control pigs in experiment 1.

The remaining pigs in experiments 1 and 2 were euthanized at selected time points after infection, and the tissues were processed for other studies to be reported at a later date.

Experiment 3. Thirty 17-day-old pigs were used in this experiment, which was designed to quantify the amount of LPMV in tissues and fluids of infected animals and the virus excretion patterns of LPMV-infected pigs. These animals were housed in accommodation similar to that described for the pigs in experiments 1 and 2 and inoculated with LPMV using the same virus pool and inoculation procedures. Rectal and nasal swabs were collected from 6 pigs on each of days 1, 2, 3, 4, 5, 7, 8, 9, 11, and 14 after inoculation and processed for virus isolation. In addition, 2 pigs were euthanized on each of these days, and feces, urine, and nasal mucosa were processed for titration in cell cultures to quantify virus load. Exact 10% suspensions (1 g of specimen in 9 ml diluent) were made from these samples, and the supernatants, obtained following centrifugation, were titrated by serial 10-fold dilution before inoculation of a 0.1-ml volume per dilution into each of 4 PK/15 cell cultures. These cultures were processed for virus isolation as described above and titration end points were read using HA on cell lysate preparations.

Blood samples were obtained from all pigs used in experiments 1, 2, and 3 immediately prior to euthanasia, and serum and buffy coat samples were prepared and processed for serologic studies and/or virus isolation.

Results

Experiment 1. Clinical signs of LPMV infection were first noted in the pigs used in this experiment at 5 days after inoculation with the virus pool. These signs included a mild increase in respiratory rate, dullness in all inoculated animals, and trembling and incoor-

Table 1. Results* of virus isolation (VI) and immunofluorescent staining of cryostat sections (IF) of tissues from pigs experimentally infected with LPMV at 3 days of age.

Tissues	Days after inoculation													
	1		2		3		4		5		7		8	
	VI	IF	VI	IF	VI	IF	VI	IF	VI	IF	VI	IF	VI	IF
Cerebrum	−	−	−	−	−	−	−	−	−	−	+	−	+	+
Midbrain	−	−	−	−	−	−	−	−	+	+	+	+	+	+
Medulla oblongata	−	−	−	−	−	−	−	−	+	−	−	−	+	+
Cervical spinal cord	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Thoracic spinal cord	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Lumbar spinal cord	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Cerebellum	−	−	−	−	−	−	−	−	+	−	+	+	−	−
Olfactory bulb	−	−	−	−	−	−	−	+	+	+	+	+	+	+
Trigeminal nerve	−	−	−	−	−	−	+	+	+	+	+	−	−	+
Optic nerve	−	−	−	−	−	−	+	+	−	+	−	−	−	−
Eye	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Trachea	+	−	+	−	−	−	+	−	−	−	+	−	+	−
Nasal mucosa	+	−	+	+	+	−	+	−	−	−	−	+	−	−
Bronchial mucosa	+	+	+	+	−	−	−	−	+	+	−	+	+	+
Lung	−	−	+	+	+	+	+	+	+	+	+	+	+	+
Tonsil	+	−	+	−	+	−	−	−	+	+	+	+	+	+
Thymus	−	−	−	−	−	−	+	+	−	+	−	+	+	−
Spleen	−	−	−	−	−	−	−	−	+	+	−	−	−	−
Bone marrow	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Axillary lymph node	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Bronchial lymph node	−	−	−	+	−	−	−	−	−	+	−	−	−	−
Inguinal lymph node	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Mesenteric lymph node	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Parotid lymph node	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Retropharyngeal lymph node	−	−	−	−	−	−	−	−	ND	−	−	−	−	+
Testis	ND	ND	NA	NA	NA	NA	−	−	ND	ND	NA	NA	−	−
Epididymis	ND	ND	NA	NA	NA	NA	−	−	ND	ND	NA	NA	−	−
Ovary	ND	ND	−	−	−	−	NA	NA	ND	ND	−	−	NA	NA
Salivary gland	−	−	−	−	−	−	−	−	+	−	−	−	+	−
Liver	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Kidney	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Heart	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Muscle	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Urinary bladder	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Pancreas	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Duodenum	−	−	−	−	−	−	+	+	−	−	+	−	+	−
Jejunum	−	−	−	−	−	−	−	−	−	−	+	−	−	+
Ileum	−	−	−	−	−	−	+	+	−	−	+	−	+	−
Cecum	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Colon	−	−	−	−	−	−	−	−	+	+	−	−	−	−
Feces	−	NA	ND	NA	−	NA	−	NA	−	NA	−	NA	−	NA
Buffy coat	−	NA	−	NA	−	NA	−	NA	−	NA	−	NA	−	NA
Serum	−	NA	−	NA	−	NA	−	NA	−	NA	−	NA	−	NA

* + = LPMV isolated or detected by IF; − = no LPMV isolated or detected by IF; ND = not determined; NA = not applicable.

dination in a few animals. Clinical signs in all inoculated animals progressed rapidly from 5 days and were characterized by severe respiratory distress, rigidity, arched back, and severe muscle tremors in all animals by 7 days after inoculation. By 8 days after inoculation, all experimentally infected animals were either dead or moribund and were euthanized. No clinical signs were observed in the control animals.

The results of virus isolation studies and IF staining of cryostat sections of tissues from these experimentally infected pigs are presented in Table 1. In general, virus isolation was more sensitive than IF staining of cryostat sections for the detection of LPMV. In total, LPMV was isolated from, or antigen was detected in, 72 tissues. Of these, 32 (44.4%) were positive by virus isolation and IF, 28 (38.8%) were positive by virus isolation only, and 12 (16.6%) were positive by IF only. For the majority of specimens processed for virus isolation, LPMV was recovered after inoculation of original material and 1 passage in cell cultures for 6 days.

Table 2. Results* of virus isolation (VI) and immunofluorescent staining of cryostat sections (IF) of tissues from pigs experimentally infected with LPMV at 17 days of age.

Tissues	Days after inoculation																			
	1		2		3		4		5		7		8		9		11		14	
	VI	IF	VI	IF	VI	IF	VI	IF	VI	IF	VI	IF	VI	IF	VI	IF	VI	IF	VI	IF
Cerebrum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Midbrain	–	–	–	–	–	–	–	–	–	–	+	–	+	–	+	+	+	+	–	–
Medulla oblongata	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cervical spinal cord	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–
Thoracic spinal cord	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
Lumbar spinal cord	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
Cerebellum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Olfactory bulb	–	–	–	–	+	+	+	–	+	+	+	+	+	+	+	+	–	+	–	+
Trigeminal nerve	–	–	–	–	–	–	+	–	–	–	ND	ND	–	–	+	–	–	–	–	ND
Optic nerve	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	ND	ND
Eye	–	–	–	–	ND	ND	+	–	–	–	ND	ND	–	–	–	–	–	–	–	–
Trachea	+	–	+	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
Nasal mucosa	–	+	–	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–
Lung	+	–	+	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–
Tonsil	+	–	–	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–
Thymus	–	–	–	–	–	–	+	–	+	–	+	–	+	–	–	–	–	–	ND	ND
Spleen	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Bone marrow	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Bronchial lymph node	–	–	+	+	–	–	–	+	–	–	–	ND	–	–	–	–	–	–	–	–
Mesenteric lymph node	–	–	–	–	+	–	–	–	+	–	+	+	+	+	+	+	–	–	–	–
Parotid lymph node	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
Testis	NA	NA	–	–	–	–	NA	NA	+	ND	NA	NA	NA	NA	–	–	NA	NA	–	–
Epididymis	NA	NA	–	–	–	–	NA	NA	–	–	NA	NA	NA	NA	–	–	NA	NA	–	–
Ovary	–	–	NA	NA	NA	NA	–	–	NA	NA	–	–	+	–	NA	NA	–	–	NA	NA
Liver	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Kidney	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Heart	ND	ND	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Muscle	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pancreas	–	–	–	–	–	–	–	+	–	–	–	–	+	+	+	+	–	+	–	–
Duodenum	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–
Jejunum	–	–	–	–	–	–	–	–	+	+	–	–	–	+	–	–	–	–	–	–
Ileum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cecum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Colon	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Feces	–	NA	–	NA	–	NA	–	NA	–	NA	+	NA	–	NA	–	NA	–	NA	–	NA
Buffy coat	–	NA	–	NA	–	NA	–	NA	ND	NA	–	NA	–	NA	–	NA	–	NA	ND	NA
Serum	–	NA	+	NA	–	NA	–	NA	ND	NA	–	NA	–	NA	–	NA	–	NA	–	NA

* + = LPMV isolated or detected by IF; – = no LPMV isolated or detected by IF; ND = not determined; NA = not applicable.

Figure 1. Immunofluorescent staining of LPMV antigen in cryostat tissue section of bronchial mucosa from a pig inoculated at 3 days of age and euthanized 5 days later. Note immunostaining of LPMV antigen in brochiolar epithelial cells.

LPMV was consistently recovered from, or LPMV antigen was demonstrated in, the respiratory tract (nasal mucosa, trachea, bronchial mucosa, lung) (Fig. 1). With the exception of day 4, LPMV was also isolated from or demonstrated in tonsil throughout the experiment. In contrast, LPMV was recovered from, or demonstrated in, a range of central nervous system (CNS) tissues (Fig. 2) only in the later stages of the infection (from day 4 onwards) and only 2 isolates were recovered from lymph nodes. LPMV was isolated from, or LPMV antigen was demonstrated in, intestinal tissues also in the later stages of the experiment and only sporadically. No virus was isolated from feces, serum, or buffy coat samples taken from these pigs.

Experiment 2. Clinical signs observed in the pigs used in this experiment following inoculation with LPMV were less severe than those observed in the pigs inoculated at 3 days of age in experiment 1. Mild nervous and respiratory signs were first observed in approximately 30% of these pigs at 6 days after inoculation, and by 8 days after inoculation a few pigs were moribund. Most pigs (approx 60%) within this group appeared clinically normal for the duration of the experiment; however, serology results indicated that all pigs had been infected (data not shown). No clinical signs were observed in the control pigs.

The results of virus isolation and IF staining of cryostat sections of tissues from these pigs are presented

Figure 2. Immunofluorescent staining of LPMV antigen in cryostat tissue section of olfactory bulb from a pig inoculated at 3 days of age and euthanized 5 days later. Note distribution of LPMV antigen throughout the section as discrete intracytoplasmic inclusions.

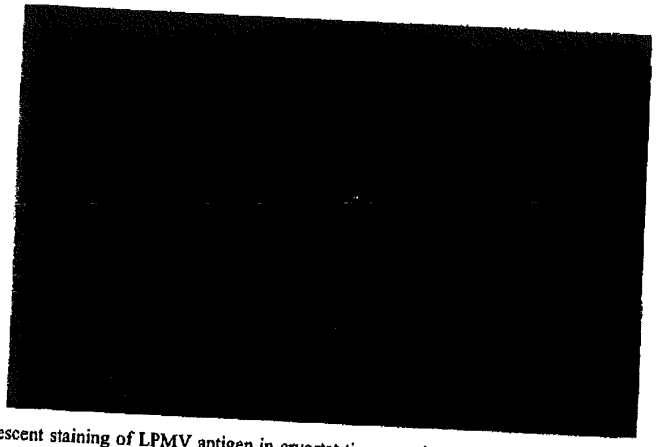

Figure 3. Immunofluorescent staining of LPMV antigen in cryostat tissue section of midbrain from a pig inoculated at 17 days of age and euthanized 9 days later. Note limited distribution of LPMV antigen (cf. Fig. 2).

in Table 2. As in experiment 1, virus isolation was more sensitive than IF as a method of detecting LPMV. Of the 70 tissues where LPMV was isolated or antigen was detected, 23 (32.9%) were positive by both methods, 31 (44.3%) were positive by virus isolation only, and 13 (18.6%) were positive by IF only. Three tissues (4.2%) not tested by IF were positive by virus isolation only. Virus was recovered from, or demonstrated in, 1 or more tissue samples from the respiratory tract (trachea, nasal mucosa, lung) or from tonsil throughout the experiment, with the exception of the pig euthanized 14 days after inoculation. In contrast, virus isolation from CNS tissues was limited and confined to the midbrain (Fig. 3) and olfactory bulb. However, discrete foci of LPMV antigen were demonstrated in cervical spinal cord from the pig euthanized 5 days after inoculation and in cervical, thoracic, and lumbar regions of spinal cord from the pig euthanized 7 days after inoculation. Several isolates of LPMV were made from lymph nodes (bronchial, mesenteric, parotid) throughout the experiment, and virus was also recovered from ovary (day 8 after infection) and testis (day 5 after infection). In addition, LPMV was demonstrated in tissue sections of pancreas on days 4, 8, 9, and 11 after inoculation (Fig. 4). Only 3 isolates of LPMV were recovered from intestinal tissues, and only 1 isolate was recovered from feces. LPMV was isolated from the serum sample taken from the pig euthanized 2 days

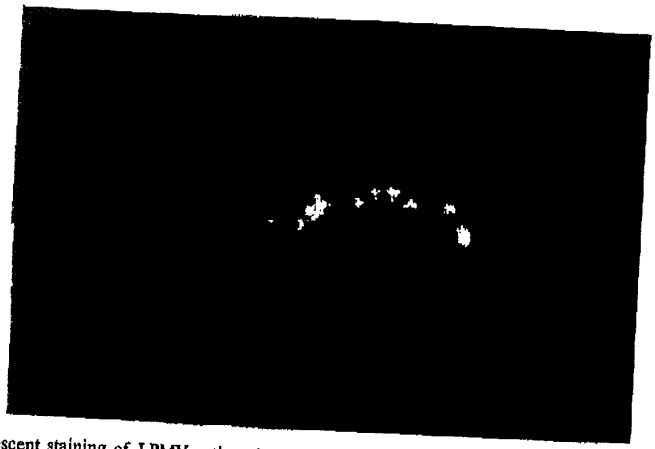

Figure 4. Immunofluorescent staining of LPMV antigen in cryostat tissue section of pancreas from a pig inoculated at 17 days of age and euthanized 8 days later.

Table 3. Results* of LPMV isolation from nasal and rectal swabs taken from pigs following experimental infection with LPMV.

Pig no.	Swab	Days after inoculation									
		1	2	3	4	5	7	8	9	11	14
265	nasal	–	–	–	–	–	–	+	ND	ND	ND
265	rectal	–	–	–	–	–	–	–	ND	ND	ND
274	nasal	–	–	+	–	–	–	–	–	–	–
274	rectal	–	–	–	–	–	–	–	–	–	–
283	nasal	–	–	+	+	+	+	–	–	–	–
283	rectal	–	–	–	–	–	+	–	–	–	–
287	nasal	–	–	+	–	+	+	+	–	–	–
287	rectal	–	–	–	–	–	+	–	–	–	–
289	nasal	–	+	+	+	+	+	–	–	–	–
289	rectal	–	–	–	–	–	–	–	–	–	–
296	nasal	–	–	+	–	+	–	–	+	–	–
296	rectal	–	–	–	–	–	–	–	+	–	–

* + = LPMV isolated in cell culture; – = no LPMV isolated in cell culture; ND = not determined (animal died).

after infection. This was the only isolate of LPMV from serum and/or buffy coat samples taken from throughout experiments 1, 2, and 3.

No LPMV was isolated from or demonstrated in tissue samples from control animals in experiments 1 and 2.

Experiment 3. The results of virus isolation tests on rectal and nasal swabs taken from 6 pigs from this group are presented in Table 3. LPMV was recovered sporadically from a total of 18 nasal swabs taken from day 2 until day 9 after inoculation, with the majority of the isolations occurring between 3 and 7 days after inoculation. LPMV was recovered from only 3 of the rectal swabs taken throughout the experiment.

The results of titrations of LPMV infectivity on feces, urine, and nasal mucosa tissues taken at necropsy from pigs in this group are presented in Table 4. The titers obtained following titration of 10% suspensions of samples of nasal mucosa were consistently $< 10^{2.00}$ $TCID_{50}$/0.1 ml, with the exception of 1 sample from a pig euthanized 5 days after infection ($10^{3.25}$ $TCID_{50}$/0.1 ml). Very little virus was recovered from fecal samples taken from necropsied animals throughout this experiment. Titers up to $10^{2.75}$ $TCID_{50}$/0.1 ml of LPMV were recorded in urine samples recovered from pigs euthanized between 5 and 11 days after inoculation.

Discussion

This is the first detailed sequential study of virus distribution in tissues and body fluids and virus excretion following experimental inoculation of pigs with LPMV by a natural route. LPMV is a comparatively newly recognized infectious agent, and the results obtained constitute an important aspect of its character-

Table 4. Virus titers detected in 10% suspensions* of nasal mucosa, feces, and urine samples from each of 2 pigs (A, B) euthanized at each time point after experimental infection with LPMV at 17 days of age.

Sample	Days after inoculation																			
	1		2		3		4		5		7		8		9		11		14	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Nasal mucosa	–	+	–	+	–	0.75	+	0.75	3.25	1.25	0.75	1.25	0.50	2.00	2.00	–	–	–	–	–
Urine	–	–	–	–	ND	ND	–	–	–	0.50	1.00	0.75	2.75	2.25	1.25	1.25	+	ND	ND	ND
Feces	–	–	–	–	–	–	–	–	–	+	–	+	0.75	–	–	–	–	–	–	–

* Log_{10} $TCID_{50}$/0.1 ml. + = LPMV recovered from undiluted sample only (titer $< 10^{0.50}$ $TCID_{50}$/0.1 ml); – = no virus recovered; ND = not determined.

ization. Experimental infections of small numbers of 1-day-old pigs with LPMV by intranasal, intracranial, and intratracheal routes have been previously reported.[9] LPMV was consistently recovered from brain and tonsil and occasionally from the lung, blood, spleen, liver, kidney, retropharyngeal lymph node, and turbinates.

The results of the present study confirm field observations that clinical signs following LPMV infection are dependent on the age of the pigs. In 3-day-old pigs, experimental infection with LPMV induced severe neurologic signs in all animals from 6 days after infection until eventual death at 8 days after infection. However, only approximately 30% of the pigs experimentally infected with LPMV at 17 days of age exhibited neurologic signs. The remaining animals appeared clinically normal throughout the duration of the experiment. No clinical signs were seen in any of 16 30-day-old pigs experimentally infected with LPMV (data not shown).

Both virus isolation and IF staining of cryostat sections were used in the present experiments to establish the sites of replication of LPMV following experimental infection. In general, virus isolation was more sensitive than IF staining. This difference is probably at least partially attributable to the very localized nature of the sites of replication of LPMV within tissues. In some tissue sections examined during this study, only a single focus of infection comprising 1 or 2 infected cells was observed. This tissue localization of LPMV replication could contribute to false-negative results, emphasising the desirability of using a combination of both techniques to increase accuracy of diagnosis.

Examination of a wide range of tissue samples from pigs sequentially euthanized in experiments 1 and 2 revealed a number of similarities in virus distribution in these different aged animals. However, differences in the distribution of virus in tissues from both age groups were also noted. Virus replication in the respiratory tract and tonsil from 1 day after inoculation was a consistent finding in both groups of pigs, indicating that the primary site of replication of LPMV may be located in these tissues. In contrast, significant differences were noted in virus distribution in CNS tissue following experimental infection of the 2 groups of pigs. Following inoculation of 3-day-old pigs with LPMV, virus was recovered from and viral antigen was demonstrated in a wide range of CNS tissue from 4 days after inoculation until the end of the experiment (8 days after inoculation). Following inoculation of 17-day-old pigs with LPMV, virus was only consistently recovered from and/or demonstrated in samples of midbrain and olfactory bulb from 3 to 11 days after inoculation. These differences in virus distribution within the CNS are related to the age of the pigs at the time of infection and may reflect differences in the dissemination of virus from the primary site of replication to the CNS. In older pigs, LPMV invasion of the CNS may occur in a manner similar to that recorded for low and moderately virulent strains of pseudorabies disease virus.[3] Virus replication may initially occur in the nasal mucosa and spread to the CNS via the trigeminal and olfactory nerves and subsequently remain localized in the olfactory bulbs and midbrain. In younger pigs, the concurrent appearance of LPMV throughout the CNS at 5 days after experimental infection and the isolation of LPMV from samples of trigeminal nerve and olfactory system from 4 days after inoculation could indicate a dual method of spread of the virus to the CNS. This route would involve primary replication of the virus in the upper respiratory tract or tonsil followed by movement through the trigeminal and olfactory nerves combined with a viremia and passage across an immature blood-brain barrier. The isolation of LPMV from only 1 of the serum and buffy coat samples taken from experimentally infected pigs during this study indicates that an extremely transient and/or low level viremia occurs following LPMV infection.

The virus excretion studies reported here indicate that excretion via the respiratory tract and in urine are important methods of spread of LPMV. Virus was recovered more consistently from nasal swabs (18) than from rectal swabs (3) from the 6 animals sampled over a 14-day period after inoculation. In addition, only minimal amounts of virus were recovered from 3 of the 20 fecal samples collected at necropsy from pigs in experiment 3; no virus was isolated from the other 17 samples. In contrast to this finding, LPMV was isolated from 12 of the 20 samples of nasal mucosa taken from these pigs and was consistently recovered from all the nasal mucosa samples taken from the pigs euthanized from day 4 to day 8 after inoculation. LPMV was also recovered at comparatively high titers from urine samples from pigs in experiment 3 euthanized between days 5 and 9 after infection, with maximum titers of virus recovered at day 8 after inoculation. Although the maximum titer of LPMV obtained following titration of urine from an infected pig was only $10^{2.75}$ $TCID_{50}$/0.1 ml, this concentration represents the amount of virus detected in 0.1 ml of a 1/10 dilution of a urine sample and, when adjusted to reflect the expected titers of virus found in the volumes of urine excreted by a pig, represents a substantial source of infectious LPMV particles.

The results reported here provide important information for the elucidation of the pathogenesis and epidemiology of LPMV infection in pigs. More detailed studies utilizing immunostaining of tissues at the light and electron microscopic level are in progress to fully

characterize the cell types involved in the pathogenesis of this virus. In addition, the establishment of a consistent and well-characterized experimental model for infection of pigs with LPMV by natural routes would be useful for the study of paramyxovirus infections of the respiratory, reproductive, and central nervous systems.

Acknowledgement

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Sources and manufacturers

a. Quil A, Superfos Biosector, Denmark.
b. Nestle UK, Croydon, UK.

References

1. Berg M, Hjertner B, Moreno-Lopez J, Linne T: 1992, The P-gene of the porcine paramyxovirus LPMV encodes three possible polypeptides P, V and C: the P protein mRNA is edited. J Gen Virol 73:1195-1200.
2. Berg M, Sundquist A, Moreno-Lopez J, Linne T: 1991, Identification of the porcine paramyxovirus LPMV matrix protein gene: comparative sequence analysis with other paramyxoviruses. J Gen Virol 72: 1045-1050.
3. Kluge JP, Beran GW, Hill HT, Platt KB: 1992, Pseudorabies (Aujeszky's disease). *In:* Diseases of swine, ed. Leman AD, Straw BE, Mengling WL, et al., pp. 313-323. Iowa State University Press, Ames, IA.
4. Linne T, Berg M, Bergvall AC, Moreno-Lopez J: 1992, The molecular biology of porcine paramyxovirus LPMV. Vet Microbiol 33:263-273.
5. McNeilly F, Allan GM, Moffet D, McNulty MS: 1991, Detection of chicken anemia agent in chickens by immunofluorescence and immunoperoxidase staining. Avian Pathol 20:125-132.
6. McNulty MS, Allan G: 1984, Applications of immunofluorescence in veterinary viral diagnosis. *In:* Recent advances in virus diagnosis, ed. McNulty MS, McFerran JB, pp. 15-26. Martinus Nijhoff, The Hague.
7. Moreno-Lopez J, Correa-Giron P, Martinez A, Ericsson, A: 1986, Characterization of a paramyxovirus isolated from the brain of a piglet in Mexico. Arch Virol 91:221-231.
8. Stephano HA, Gay GM: 1986, El sindrome del ojo azul. Una nueva enfermedad en cerdos associada a un paramyxovirus. Vet Mex 17:120-122.
9. Stephano HA, Gay GM, Ramirez TC: 1988, Encephalomyelitis, reproductive failure and corneal opacity in pigs associated with a new paramyxovirus infection (blue eye). Vet Rec 122:6-10.
10. Stephano HA, Gay GM, Ramirez TC, Maqueda AJJ: 1981, Estudio de un brote de encefalitis en lechones por un virus hemoaglutinante. Mem Congr Assoc Mex Vet Esp Cerdos 17: 43.
11. Sundquist A, Berg M, Hernandez-Jaurequi P, et al.: 1990, The structural proteins of a porcine paramyxovirus (LPMV). J Gen Virol 71:609-613.
12. Sundquist A, Berg M, Moreno-Lopez J, Linne T: 1992, The hemagglutinin-neuraminidase gene of the porcine paramyxovirus LPMV: comparison with other paramyxoviruses revealed the closest relationship to simian virus 5 and mumps virus. Arch Virol 122:331-340.

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CASE REPORT

An 18 week fetus with Elejalde syndrome (acrocephalopolydactylyous dysplasia)

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We describe a fourth case of Elejalde's syndrome (acrocephalopolydactylous dysplasia) in an 18 week fetus which had the typical features of craniosynostosis, gross oedema, short limbs, postaxial polydactyly, redundant connective tissue and cystic renal dysplasia.

Keywords: postaxial polydactyly, short limbs, redundant connective tissue, craniosynostosis, autosomal recessive inheritance

Introduction

Elejalde *et al.* reported in 1977 a new multiple congenital abnormality syndrome in two sibs, born to parents who were first cousins. The mother had a total of 13 pregnancies, of which three ended as first trimester spontaneous abortions, and 10 (eight females and two males) were liveborn, of whom the last two, a male and a female, were affected. The clinical features included high birth weight, gross oedema, short neck with redundant skin folds, short limbs, postaxial polydactyly and acrocephaly with a closed anterior fontanelle. In addition, both sibs had an omphalocele and lung hypoplasia. Histologically, there was pancreatic fibrosis, cystic renal dysplasia and increased connective tissue of intestinal wall, pancreas, kidneys, gall bladder and skeletal muscles. Lurie *et al.* (1991) also reported a case, a male, born to non-consanguineous Russian parents. Their patient had premature parietal synostosis, a cavernous haemangioma in the neck, bilateral postaxial polydactyly of fingers, cystic renal dysplasia and severe hypoplasia of the small intestine and colon.

We report an 18 week male fetus with the features of Elejalde syndrome.

Case report

The mother, a primigravida, was aged 20 years and the father 19 years at the birth. Ultrasound examination at her first antenatal visit at 14 weeks gestation, showed a single fetus with a BPD of 28 mm which was equivalent to the period of amenorrhoea. At the second antenatal visit at 18 weeks gestation, the ultrasound examination showed a grossly abnormal fetus with a large

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Fig. 1. 18 week male fetus showing gross oedema, preaxial polydactyly and short limbs.

cystic loculated area at the base of the skull, extending around the neck. The spine was normal. The limbs appeared short; length of limbs was equivalent to 14 weeks gestation. Fluid was present in the chest and the abdomen. The parents decided to terminate the pregnancy. The male fetus weighed 240 g (161±91), with foot length 20 mm (26±0.8), crown-rump length 16.5 cm (13±3), crown-heel length 21 cm (19±4) and head circumference 14.5 cm; the figures in brackets indicate mean value ± 2SD at 18 weeks gestation (Singer *et al.*, 1991). These measurements are mostly at the upper limit of normal for 18 weeks gestation.

The body (Fig. 1) was grossly swollen, especially the nuchal region and around the neck. The face was so swollen that the palpebral fissures appeared slit-like. The nose was upturned with a long philtrum and the low set ears which had pointed helices were swollen. There was micrognathia. The anterior fontanelle was closed. The limbs were short; unfortunately, no X-rays were available. There was bilateral postaxial polydactyly of the fingers and toes. All major internal organs were present. Microscopically, there was increased interstitial connective tissue in the pancreas, kidneys, intestine and heart. The kidneys also showed dilatation of Bowman's space in the glomeruli and cystic dilatation of the renal tubules (Fig. 2). Spleno-pancreatic fusion was present (Fig. 3). In addition to the proliferation of connective tissue, there was increased proliferation of nerve fibres in many organs (Fig. 4).

During the mother's second pregnancy, an ultrasound examination at 19 weeks showed a single fetus with a BPD of 48 mm which was equivalent to the period of gestation. The femur length was 32 mm, humerus 30 mm and radius 25 mm, consistent with a 19 week pregnancy. Five fingers

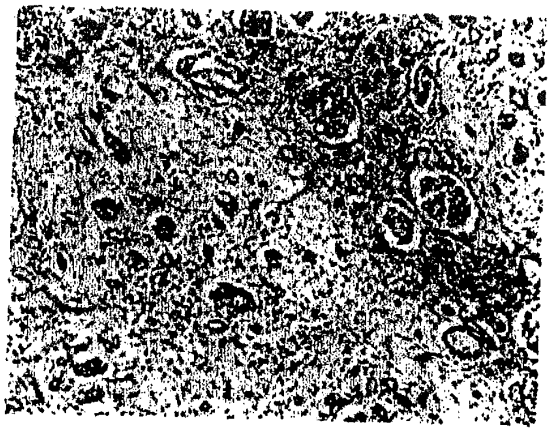


Fig. 2. Kidney showing dilated renal tubules and proliferation of connective tissue.

Fig. 3. Splenopancreatic fusion without any definite delineation.

Fig. 4. Pancreas stained with S100 immunochemical stain demonstrating the proliferation of nerve fibres.

were present on each hand. Subsequently she delivered a normal infant. The only family history of note is that mother's paternal aunt has acrocephalosyndactyly (Apert syndrome). Both parents are clinically normal.

Discussion

Table 1 summarizes the clinical features and other details of acrocephalopolydactylous dysplasia (Elejalde syndrome). Our patient had most of the clinical features present in the previous patients (Elejalde *et al.* 1977; Lurie *et al.*, 1991). Although no radiological investigations were available, craniosynostosis was inferred from the absence of the anterior fontanelle. The limb lengths measured by ultrasound were equivalent to a 14-week rather than an 18-week fetus. In any future cases, there is a need for detailed radiology. Interestingly, the paternal aunt of our patient's mother had acrocephalosyndactyly (Apert syndrome), an autosomal dominant disorder, with craniosynostosis as a feature. The family reported by Elejalde *et al.* (1977) had a pattern of inheritance consistent with an autosomal recessive trait; in addition to the two affected sibs, a brother of the father, married to a first cousin, had eight children, one of whom allegedly was affected by the condition. The recognition of Elejalde's syndrome is important for genetic counselling. Our family was advised, on the basis of autosomal recessive inheritance of a one in

Table 1. Clinical features of Elejalde syndrome

Characteristics	*Elejalde* et al. *(1977)*		*Lurie* et al. *(1991)*	*Present case*
	Case 1	*Case 2*		
Sex	M	F	M	M
Birth weight (g)	7500	4300	4400	240
Gestation (weeks)	34	34	33	18
Placental weights (g)	3500	1500	NR	175
Maternal age (years)	32	33	33	20
Paternal age (years)	40	41	38	19
Consanguinity	+	+	–	–
Origin/country	USA	USA	Russia	North Irish
Survival (min)	Few	15	Few	IA
Length (cm)	51	39	NR	21
OFC (cm)	39	35	NR	14.5
Craniosynostosis	+	+	+	+
Absent anterior fontanelle	+	+	NR	+
Oedematous	+	+	+	+
Postaxial polydactyly	–	+	+	+
Micromelia	+	+	NR	+
Congenital heart defect	+[b]	–	–	–
Omphalocele	+	+	–	–
Renal dysplasia	+	+	+	+
Anomaly spleen	–	+	+	+[a]
Chromosome analysis	NE	46,XX	NR	46,XY

NR, not recorded; NE, not examined; IA, induced abortion; [a], splenopancreatic fusion; [b], patent ductus arteriosus

four recurrence risk. During the second pregnancy, ultrasound examination at 19 weeks gestation showed normal limb lengths, all digits and a normal sized cranium. Subsequently, she delivered a normal infant.

The aetiology of Elejalde syndrome is unknown. In the previously reported cases, excessive amounts of connective tissue were noticeable in almost every organ except the central nervous system. This was most noticeable in the subcutaneous tissue, media of blood vessels and in the interstitium of the pancreas and liver. Our patient also showed this excessive proliferation of connective tissue. Cell kinetic studies showed that fibroblasts from Elejalde syndrome patients completed the whole cycle in 63% of normal cell cycle time (Elejalde *et al.*, 1977). This syndrome is an overgrowth syndrome (Cohen, 1989) but the causative factors are as yet unidentified.

Future cases of this syndrome should have detailed radiology and cell kinetic studies.

References

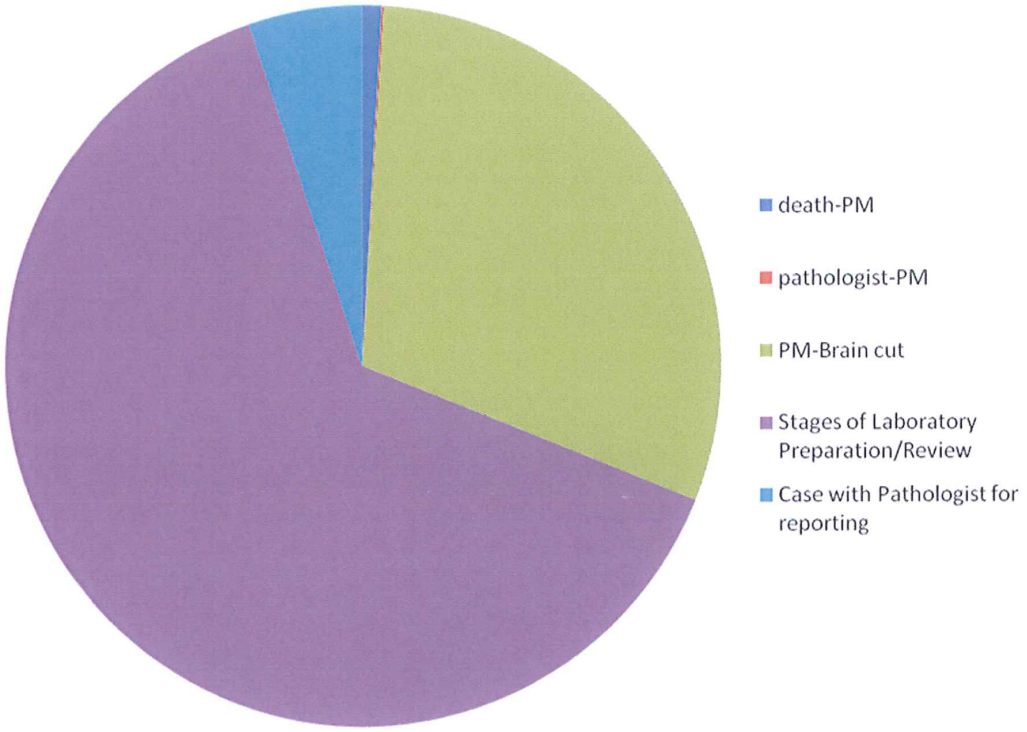
Cohen MM Jr (1989): A comprehensive and critical assessment of overgrowth and overgrowth syndromes. Elejalde syndrome. Adv Hum Genet **18**: 181–303.

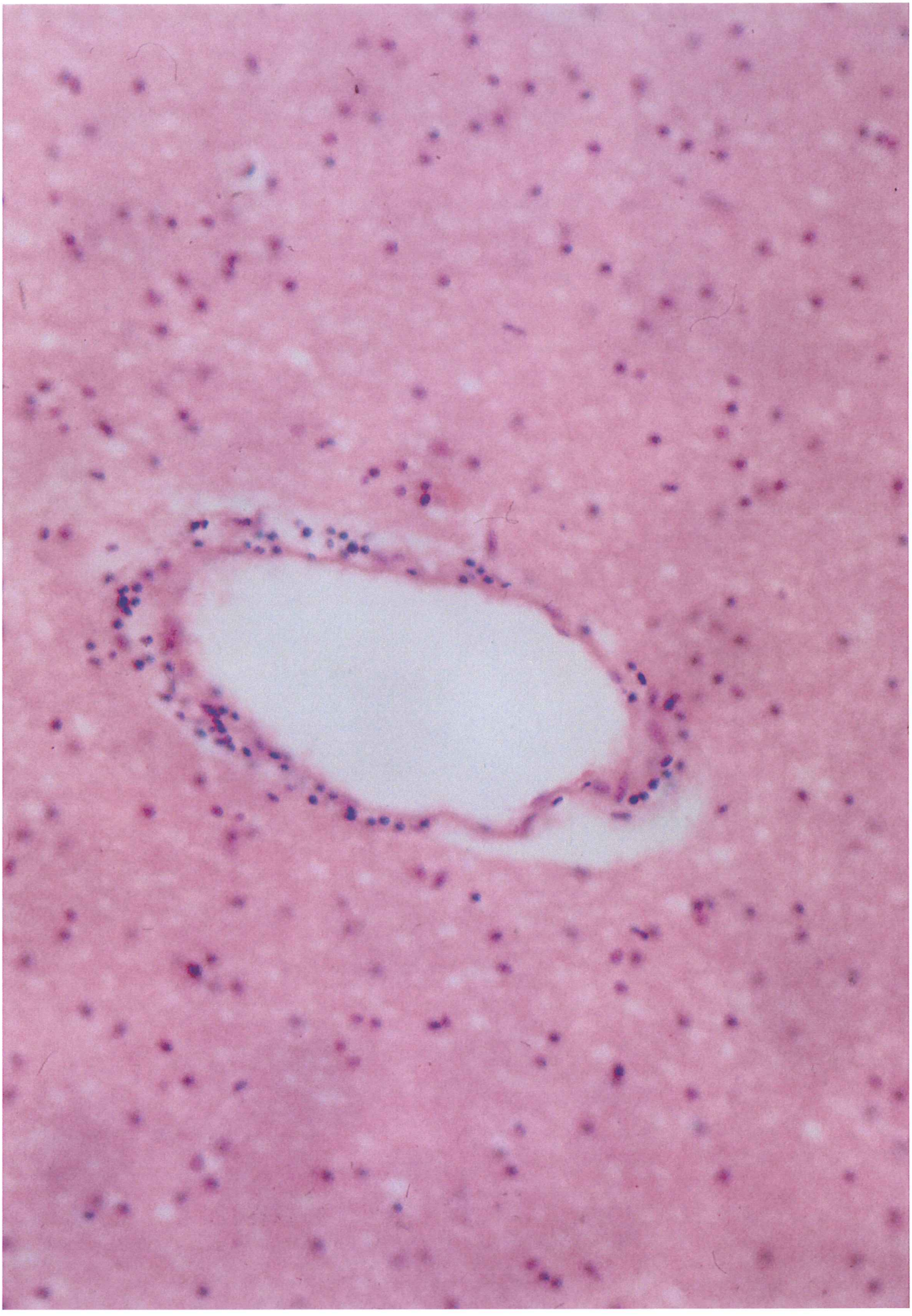
Elejalde BR, Giraldo C, Jimenez R, Gilbert EF (1977): Acrocephalopolydactylous dysplasia. Birth Defects: Original Article Series, Volume XIII, **3B**, 53–67.

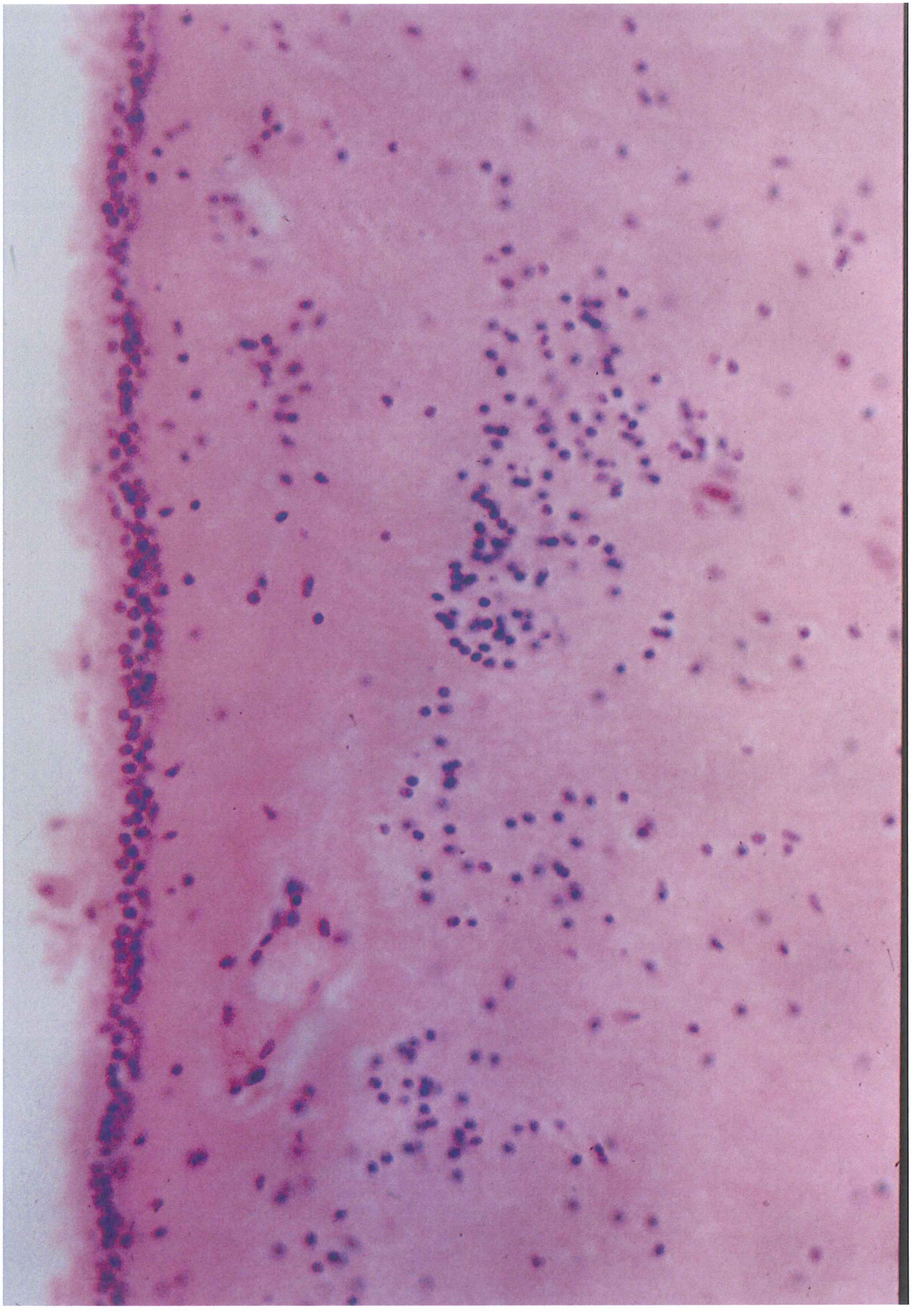
Lurie IW, Lazjuk GI, Korotkova IA, Cherstvoy ED (1991): The cerebro-reno-digital syndromes: a new community. Clin Genet **39**: 104–113.

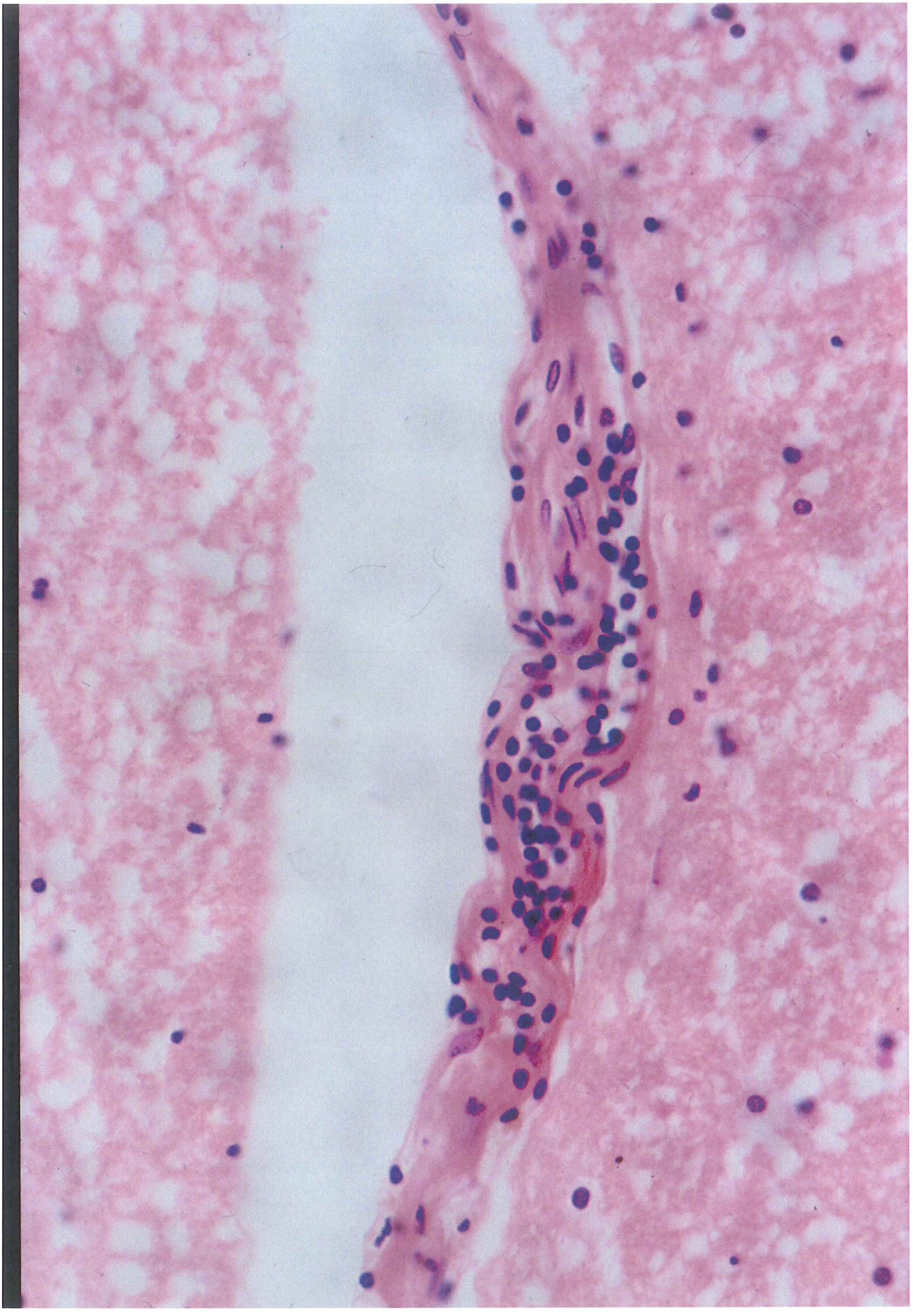
Singer DB, Sung CJ, Wigglesworth JS (1991): Fetal growth and maturation; with standards for body and organ development. In Wigglesworth JS, Singer DB (eds): "Textbook of Fetal and Perinatal Pathology." Vol 1. Blackwell Scientific Publications; pp. 11–47.

Time frame for completion of Post Mortem Report-the role of the neuropathologist. Typically the neuropathologist is directly involved in less than 10% of the reporting time.









DEPARTMENT OF NEUROPATHOLOGY

ROYAL HOSPITALS TRUST/QUEEN'S UNIVERSITY

The following cases will be discussed at the Neuropathological session on **Tuesday 27th April at 9.15 am in the Sir Samuel Irwin Lecture Theatre.**

POST MORTEM REPORTS:

P.M. No:	Hospital No.	Name	Clinician	Pathologist
NPPM		,		Dr Mirakhur

BIOPSY REPORTS:

				Dr Herron
				Mr I Wallace
				Dr Herron

NB: PLEASE REQUEST ONE OF YOUR TRAINEES TO PRESENT THE CASE IF YOU CANNOT ATTEND.

COFFEE WILL BE AVAILABLE AFTER THE MEETING