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The microbiological investigation of sudden unexpected death in infancy

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INTRODUCTION

Sudden unexpected death in infancy (SUDI) is simply defined as the death of an infant that is sudden and is unexpected. If a detailed post-mortem examination fails to reveal an adequate explanation for death then the term 'sudden infant death syndrome' (SIDS) is used. If the autopsy does not reveal an explanation for death, but there are suspicious features, the term 'unascertained' is often applied. The difference between SIDS, which legal authorities will regard as natural disease, and unascertained is, therefore, related to the level of suspicion. The latter term can cause distress to parents and lead to unnecessary inquests. It should be used sparingly. In strict logic, of course, the difference between 'I don't know' (SIDS) and 'I don't know' (unascertained) is unascertained and unascertainable.

The age distribution of SUDI and SIDS is the most consistent and characteristic feature of sudden infant death. The risk of SUDI and SIDS is low in the first few days of life, the risk then rises to a peak at two to three months, followed by a rapid fall so that the condition is uncommon after six months and rare after twelve months (Fig. 1.1). This risk profile is approximately reciprocal to infant serum IgG levels, and therefore sudden death occurs when infants have least protection against common bacteria and common bacterial toxins. For this reason it is important that careful microbiological investigation is carried out in all sudden infant deaths.

There are published protocols for the microbiological investigation of these cases, with which we broadly concur [1], [2]. Certain aspects of the investigation, however, are more important than others and the time when specimens are obtained is crucial. In this chapter we intend to concentrate on aspects of the investigation, giving background information as to why the tests are needed and placing emphasis on practical procedures as well as discussing the difficult task of interpreting the results obtained.

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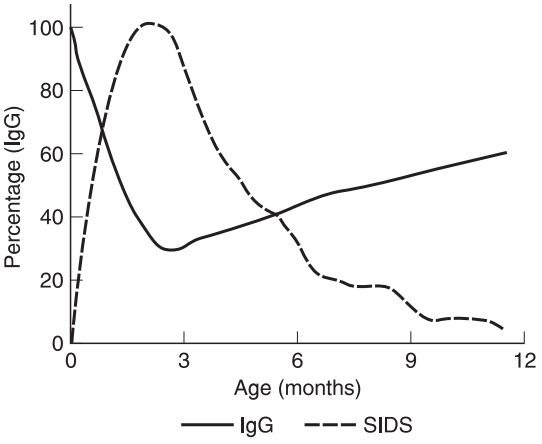

Fig. 1.1 Serum IgG levels in infancy expressed as a percentage of the adult level. The age distribution of SIDS is for cases in England and Wales between 1993 and 1995 (1311 cases).

CEREBROSPINAL FLUID

The diagnosis of meningitis in life depends on the examination of cerebrospinal fluid (CSF). In a suspected case of meningitis, CSF is obtained using an aseptic technique to avoid contamination by bacteria from the skin surface. The specimen is examined promptly; a white cell count and differential cell count are performed; protein, glucose and, in some laboratories, lactate levels are determined. The specimen is then examined for bacteria using Gram stain and bacterial culture. Part of the specimen is sent to a specialised laboratory for viral culture and some saved in case other specialised techniques are required, such as nucleic acid amplification or immunoassay to identify specific organisms.

We suggest that exactly the same approach should be used in cases of SUDI. The problem is, however, to obtain the specimen sufficiently soon after death for the investigation to be useful.

WHITE CELL COUNT

Normal CSF contains no more than four mononuclear cells per cubic mm and no polymorphs [3]. Inflammation of the meninges (meningitis) leads to protein and white cell exudation so that the white cell count rises. The rise is predominantly lymphocytic in viral infection, while polymorphs predominate in bacterial infection. A major problem of interpretation of post-mortem CSF, however, is that the mononuclear cell count rises after death in the absence of inflammation. Thus Platt *et al*. [4] found CSF counts ranged between 37 and 3250 cells per cubic mm (mean = 647 per cubic mm) in 26 cases of SIDS, in which there was no evidence of meningitis. The post-mortem intervals were stated as 2 to 28 hours. In adult autopsies the CSF pleocytosis was less marked, with a mean of 28 white cells per cubic mm (mean post-mortem interval 15 hours). In SIDS cases the typeable cells were mononuclear and consisted of 60 to 70% lymphocytes and

Table 1.1 Normal CSF protein ranges in infancy

Term	
neonate	0.2–1.7 g/l
1–30 days	0.2–1.5 g/l
30–90 days	0.2–1 g/l
3–6 months	0.15–0.3 g/l
6 months–10 years	0.15–0.3 g/l

20 to 40% macrophages. The authors did not record the presence of polymorphs. When the post-mortem interval exceeded 12 hours the cells became vacuolated and could not be identified. The CSF cell count rises with the duration of the post-mortem interval, but the rate of rise is decreased if the body is stored at 4 °C as opposed to 20 °C [3].

The mononuclear cells in the CSF are apparently derived from the lining cells of the arachnoid membranes. In the absence of inflammation polymorphs should not cross the blood–brain barrier and therefore, if polymorphs are seen, they are, in theory, an absolute indicator of inflammation. This seems to be borne out in practice as neither of the above publications [3], [4] record the presence of polymorphs in the absence of meningitis. In order to gain maximum information, therefore, a differential cell count should be performed on post-mortem CSF samples, but they would have to be obtained within a few hours of death for this to be useful. The distinction between polymorphs and mononuclear cells in samples showing degenerative changes can be difficult, and modern staining techniques, using a range of specific antisera, on cytocentrifuge preparations should be deployed (a useful research project for someone).

PROTEIN

Fishman [3] gives normal ranges for CSF protein in infancy (Table 1.1)

The blood–brain barrier is maintained in health so that the protein concentration in plasma is much higher than in CSF (i.e. 200-fold difference in adults). Meningeal inflammation, however, leads to increased permeability of meningeal vessels, and proteins leak into the CSF. The blood–brain barrier was first described in autopsy studies conducted in the nineteenth century [5]. Ehrlich and his students injected aniline dyes intravenously, the dyes became attached to albumin in blood. At autopsy it was found that the tissues were stained blue but not the CSF, which remained clear, nor the brain. Thus with rapid death and a short post-mortem interval there is no leakage of protein into the CSF in experimental animals.

There are relatively few studies of CSF protein changes in humans following death and no large systematic studies in infants [6]–[9]. The study by Mangin *et al*. [7] demonstrates a good correlation between the clinical history and the CSF protein concentration when the samples were obtained within 24 hours of death. If death was rapid, such as in cases of homicide, the CSF protein was within the normal range. If the process of dying was prolonged and associated

with inflammation and cytokine release, as in patients in intensive care, the CSF protein was raised. In the study conducted by Osuna *et al*. [9], in comparison, all the CSF protein values were raised and bore little relation to the premorbid condition. In this study, however, the samples were obtained after a mean interval of 48 hours. Thus it appears that the CSF protein estimation can be useful but only if obtained soon after death. This is another area in which more research is required.

CSF GLUCOSE AND LACTATE LEVELS

Polymorphs are anaerobic cells, when activated they metabolise glucose and cause a fall in pH and a rise in lactate levels. Thus bacterial infection is associated with a fall in CSF glucose and a rise in CSF lactate. The same changes, however, can follow death in the absence of infection and therefore these analyses are of limited value in post-mortem samples.

PRACTICAL CONSIDERATIONS

The usual practice of taking a CSF sample 24, 48 or even 72 hours after death results in loss of information. In most cases of sudden death the infants are taken urgently to A&E departments and a consultant paediatrician attends to supervise resuscitation and to decide when to desist. The consultant paediatrician should be empowered by the coroner to commence the investigation by obtaining samples for microbiology, including CSF. The sample should then be sent to the laboratory and treated urgently, as for a case of suspected meningitis in life. There is no point in getting the specimen early and then allowing it to deteriorate over several hours. The sample should be taken with full aseptic precautions to reduce the possibility of bacterial contamination. The result of the analysis will then be ready prior to the autopsy. The pathologist should also take a sample when the autopsy is undertaken, preferably by cisternal puncture (after careful cleaning of the skin with alcohol wipes) so as to obtain sufficient fluid in case further studies are required (see below). Paediatricians are skilled at taking clean samples but pathologists are better at getting large volumes of fluid.

HISTOPATHOLOGICAL CORRELATION

There is a school of thought that meningitis can be diagnosed on the basis of histological examination alone, and that therefore counting white cells in the CSF and measuring the protein level is unnecessary. In my opinion this view is misguided for the following reasons:

1. Histological examination of the meninges is 100% specific for meningitis, i.e., by definition, those without meningitis will not show lymphocytic and polymorph infiltration of the meninges. Since no biological test is both 100% sensitive and 100% specific it follows that histological examination cannot identify every case of meningitis (sensitivity is the percentage of individuals with meningitis who show diagnostic histological features of meningitis).

2. In viewing a section of meninges on microscopy we would expect to see at least one polymorph per high power field in a case of meningitis. The mean diameter of a high power field is 0.5 mm and the section thickness is 0.005 mm. Thus one polymorph per high power field is equivalent to 800 polymorphs per cubic mm of tissue. Eighty polymorphs per cubic mm of CSF is more than enough for a diagnosis of meningitis, but the equivalent (one polymorph per 10 high power fields) is not enough. Thus, simple calculations indicate that in the early stages of meningitis examination of the CSF will allow a diagnosis to be made before diagnostic changes are seen in tissue sections. The CSF white cell count is a more sensitive indicator than histology.
3. Sonnabend *et al*. [10] conducted a very careful microbiological study of 70 cases of SUDI. They found evidence of overwhelming infection in eleven. They comment that 'the post-mortem cultures were of diagnostic value, providing the sole means of identifying the cause of death in 8 (11%) of the 70 infants, in whom the presence of an infection could be established only after repeated and extensive histological investigations'. Thus, initial histological investigations were negative but further sections revealed foci of inflammation. This principle applies generally, in the earliest stages of infection the changes may be focal and missed with standard samples.

Sadler [11] reviewed 95 infant deaths examined according to a detailed protocol in which the autopsy was conducted between 2.5 and 53 hours (mean 11 hours, median 5 hours) after death. The author records 'ten (16%) of the apparent cot deaths were explained on the sole basis of unexpected positive microbiological findings, mostly meningococcal or pneumococcal meningitis and/or septicaemia'. Thus, infection, particularly meningitis, can be missed if reliance is placed on histology alone.

INTERPRETATION OF POSITIVE MICROBIOLOGICAL CULTURES

The interpretation of positive microbiological cultures from blood or CSF is fraught with problems[12]. In theory, positive cultures can be due to:

1. Bacteria invading the blood and tissues in life. This is usually associated with inflammation and in the absence of another explanation it is assumed that the bacteria cause or contribute to death.
2. Bacteria entering the blood in the agonal phase or during attempts at resuscitation.
3. Bacterial growth after death followed by tissue invasion. This is reduced but not eliminated by storage of the body at 4 °C.
4. Contamination of the samples, when they are obtained, by surface organisms. This can also be reduced, but not eliminated, by careful technique.

The following is a useful working rule:

A. A pure growth of a pathogen in the CSF in association with inflammation is regarded as the likely cause of death.
B. A pure growth of a pathogen without evidence of inflammation is regarded as a possible cause of death.

C. Other results, such as a mixed growth of organisms in the absence of inflammation, are more likely to be a consequence of mechanisms 2, 3 or 4.

If the specimen is obtained soon after death using a good technique, the chance of false positives due to mechanisms 3 and 4 is reduced.

VIROLOGY

A specimen of CSF should be sent for virological studies. This also applies to blood, upper and lower respiratory secretions and bowel contents. The interpretation of the results is even more difficult than with bacteria. Infants will encounter a number of viruses in the first year of life, and therefore their presence does not necessarily imply significant disease. Isolation of a virus, together with evidence of inflammation at an appropriate site, should be regarded as a possible explanation for death. Isolation of a virus without other evidence of disease leaves the case unexplained.

TOXICOLOGY

The idea that common bacterial toxins could have a role in the pathogenesis of SUDI and SIDS [13] has been strengthened by the discovery that over 50% of SIDS cases have detectable staphylococcal pyrogenic enterotoxins in brain and other tissues [14]. These toxins (toxic shock syndrome toxin (TSST)), and staphylococcal enterotoxins A, B and C) are only produced by staphylococci when the temperature is raised to between 37 and 40 °C. Thus, the presence of these toxins in tissues indicates production in life rather than after death. These results need to be confirmed, but there is a good case for measuring staphylococcal toxins in CSF in every case of SUDI and SIDS.

RESEARCH POINTS

1. The concept that a post-mortem mononuclear cell pleocytosis in the CSF has no pathological significance is open to challenge. The CSF post-mortem cell counts in SIDS are much higher than in adults and there is a possibility that meningitis is being missed. Enumeration of cell sub-types using antisera against the wide range of cluster differentiation antigens (CD) now recognised should aid in distinguishing blood–borne mononuclear cells from the lining cells of the arachnoid membranes.
2. Proteomics is replacing genomics as the vanguard of biological science [15]. Proteins in fluids, such as CSF, can be separated by two-dimensional electrophoresis and then analysed by mass spectroscopy, and their amino acid sequence determined. This entire process can be automated, and in the future it will be possible to recognise foreign proteins, such as bacterial toxins, and products of inflammation. This is big science, rather than the small-scale science we are accustomed to in pathology, but it should be brought to bear on the problem of SUDI.

Table 1.2 Causes of bacteraemia and meningitis in infancy

Under 1 month old	1–3 months old	Over 3 months old
Group B streptococcus	*Streptococcus pneumonia*	*Streptococcus pneumoniae*
Enterobacteriaceae	Group B streptococcus	*Haemophilus influenzae*
Listeria monocytogenes	*Neisseria meningitidis*	*Neisseria meningitidis*
Streptococcus pneumonia	*Salmonella* spp.	*Salmonella* spp.
Haemophilus influenzae	*Haemophilus influenzae*	
Staphylococcus aureus	*Listeria monocytogenes*	
Neisseria meningitidis		
Salmonella spp.		

This table is adapted from Brook [16]

BLOOD CULTURE

A blood culture should also be taken prior to the autopsy and as soon after death as possible. The specimen should be obtained by the consultant paediatrician who attends in A&E. The subsequent assessment of any bacterial growth must take into account the four possible routes of bacterial access noted in the previous section: invasion prior to death, invasion during the agonal phase or as part of resuscitation, post-mortem growth, or contamination. But if the specimen is obtained soon after death using a careful aseptic technique, the possibility of post-mortem growth and contamination is greatly reduced.

Table 1.2 lists the organisms that cause bacteraemia and meningitis in infants [16]. If one of these organisms is isolated in pure growth, then it is a likely explanation for death. If the culture produces a mixed growth of skin commensals then contamination should be suspected. A mixed growth of organisms from the gut or respiratory tract points towards an agonal ingress of bacteria. None of these rules, however, is absolute.

Correlation of the microbiological findings with histology can aid interpretation. If there is evidence of inflammation on serosal surfaces or in the liver or lung, then this increases the likelihood of genuine infection. The absence of inflammation, however, as in meningitis, does not exclude infection.

Table 1.3 shows the results of positive blood cultures obtained in Lancaster, UK from all age groups for one year ($n = 371$). Positive cultures judged to be a result of contamination are excluded. *Escherichia coli* and *Staphylococcus aureus* together account for 40% of serious infections in all age groups except infants aged 1 month to 12 months. This is in spite of the fact that colonisation of the upper airways by *S. aureus* is maximal in the first few months of life [17] and infant serum lgG levels reach their lowest levels at two to three months of age. Brook states [16] 'Most young children who develop bacteraemia are immunologically intact. The process is initiated by nasopharyngeal colonisation and followed by bacterial invasion of the blood and rare systemic dissemination. Both colonisation and bacteraemia are often associated with a preceding viral respiratory tract infection'. It appears that *S. aureus* bacteraemia and meningitis is not observed at the very time one would expect it to be most common.

Table 1.3 Clinically significant blood culture isolates in one year from Royal Lancaster Infirmary, UK ($n = 371$)

Organism	Percentage
Escherichia coli	24.5
Staphylococcus aureus	15.9
Streptococcus pneumoniae	9.2
Klebsiella pneumoniae	5.7
Enterococcus faecalis	4.0
Proteus mirabilis	2.9
Group B streptococci	2.9
Pseudomonas aeruginosa	2.4
Group A streptococci	2.4
Serratia marcescens	1.8
Enterococcus faecium	1.8
Candida albicans	1.8
Other organisms	24.7

The absence of significant *S. aureus* infection in infancy is also at variance with the finding of staphylococcal toxins in the brain of over 50% of SIDS cases [14]. Is it that staphylococcal toxaemia kills before bacteraemia occurs? Is it that termination of bacteraemia by polymorphs leads to toxin release and sudden death? Or is it that the microbiological investigation of SUDI is too cursory, and disseminated staphylococcal infection is missed or ignored?

A specimen of blood should also be obtained at autopsy for possible virological, toxicological or genetic analyses. It is best to clean the skin with alcohol wipes, then cut down to a vein with a new scalpel blade and obtain blood by venepuncture. The specimen of blood should be separated and stored.

SPLENIC CULTURE

A specimen of spleen can be obtained at autopsy for bacterial culture, and this is a useful adjunct to blood culture. The specimen should be obtained immediately the abdomen is opened. One approach is to push a swab into the spleen. A second is to cut off a small piece using a clean knife and send it for microbiology. The piece is subsequently placed in boiling water to sterilise the surface, and then splenic tissue from the centre is used for culture.

RESEARCH POINT

E. coli and *S. aureus* are common causes of bacteraemia except in infants between one and twelve months of age. Either these organisms do not cause bacteraemia in this age group, or they do, but the infection progresses rapidly to death and is missed. The observation that staphylococcal enterotoxins and *E. coli* endotoxin can interact synergistically to cause death in experimental models [18] is pertinent to answering these questions. Nucleic acid amplification techniques

(polymerase chain reaction) need to be used on blood and CSF specimens from cases of SUDI to see if the bacteria have been present, even if they cannot be grown. If blood can be obtained soon after death, then endotoxin measurements may also be useful [19].

UPPER RESPIRATORY TRACT

The nasopharyngeal bacterial flora of SIDS infants is different from that in healthy live infants matched for age, gender and season [20]. A study conducted in the 1980s, when most infants slept prone, revealed increased carriage of staphylococci, streptococci and Gram negative bacilli in infants at autopsy [20]. Although the possibility of Post-mortem change cannot be discounted as an explanation for the difference, a similar pattern is found in the early morning in infants suffering from a viral upper respiratory infection who have slept prone in the night [17]. Enterobacteriaceae, such as *E. coli* and *Klebsiella* spp., are rarely found in healthy infants, occur in less than 3% of those with clinical upper respiratory tract infections (URTI) who sleep supine, but are commonly found in the early morning in those with URTI who sleep prone, and were found in up to 45% of SIDS cases in the 1980s [17], [20].

S. aureus is found in approximately 50% of normal healthy infants in the first three months of life. The carriage rate thereafter gradually falls to 30% by 6 months of age. *Streptococcus pneumoniae*, by comparison, occurs in 5% in the first month of life and then gradually rises to 30% by 6 months. *Haemophilus influenzae* is rarely found in the first month but then rises to 10% at 6 months. Enterobacteriaceae remain under 3% throughout the first six months of life [17]. Streptococci are commonly found but group A haemolytic streptococci are rare. Other pathogens, such as meningococci and *Bordetella pertussis* are also rare.

In SUDI a pernasal swab should be taken as soon after death as possible, once again this is best obtained in A&E rather than waiting till the autopsy is undertaken. A full analysis of the nasopharyngeal flora requires examination using a wide range of culture media. In practice, most laboratories will concentrate on the identification of possible pathogens, but this should include *S. aureus* and at least partial identification of the enterobacteriaceae.

If *S. aureus* is isolated, the organism should be sent for genetic analysis to see if it produces any of the pyrogenic enterotoxins such as toxic shock syndrome toxin (TSST) and the staphylococcal enterotoxins A, B, C or D. These toxins are superantigens, their production is switched on when the temperature rises above 37 °C. They cause a polyclonal proliferation of T-lymphocytes, leading to an outpouring of cytokines, and this in turn can cause profound shock as in the toxic shock syndrome. These toxins have been found in the brain tissue of over 50% of SIDS cases, as noted above [14] and, therefore, are leading contenders for a causative role in SIDS.

In my opinion, if a toxigenic *S. aureus* is isolated, then the CSF and blood should be analysed for the respective toxin using an antibody probe. This test is not routinely available but can be performed. A positive result would point strongly to a mechanism of death based on toxic shock.

A pernasal swab, passed through the nose to touch the posterior nasopharyngeal wall, is the best method of sampling the flora of the upper respiratory tract. A throat swab is less useful overall but may give a higher isolation rate for pathogenic streptococci. Thus, either a pernasal swab alone or a pernasal swab plus a throat swab should be obtained, but not a throat swab alone.

LOWER RESPIRATORY TRACT

Microbiological investigation of the lower respiratory tract depends on samples obtained at autopsy. If the autopsy cannot be undertaken immediately the body should be stored at 4 °C. The samples should be obtained as soon as the chest is opened and before the abdomen is opened. A clean scalpel blade should be used to obtain a sample of lung, and swabs should be passed down the trachea and into the bronchi as soon as the chest contents are removed. The specimens obtained can be used for both bacteriology and virology. If, during the subsequent dissection of the lungs, a focal area of consolidation is noted, a further sample should be obtained for bacteriology, but the chance of contamination will inevitably be increased.

The results of bacterial culture must be interpreted with care. In life the upper airways have a resident bacterial flora but the lower airways do not, and therefore the presence of bacteria in the lower respiratory tract is of significance. The problem, however, is that the lower airways may be contaminated from the upper airways during attempts at resuscitation. This is where careful analysis of the flora of the upper airways can help. If the mixture of organisms in the upper and lower airways is the same, then contamination is the more likely explanation. But if there is a pure growth of a pathogen in the lower respiratory tract and a mixed growth in the upper respiratory tract, a genuine infection is more likely. Furthermore, if the same organism is found elsewhere, e.g. in the blood or CSF, then infection is clearly the most likely explanation.

Correlation of bacteriology and histology is an important, but a vexed, problem. If there is histological evidence of pneumonia and a pathogen is isolated, then the diagnosis is clear. The difficulty arises when the bacteriological findings point to infection and histology is negative. The process of inflammation is defined by the histological appearance and histology is 100% specific; therefore, it cannot be 100% sensitive or we would be denying the essential uncertainty and variability that underlies biology and pathology. Thus, there will be false negatives and cases of genuine infection will be missed if we regard histology as the gold standard. This is particularly important in SUDI, as recent evidence suggests that infants can go from being apparently well to death in under 20 minutes [21]. This does not give time for the classical signs of inflammation to arise.

THE GASTROINTESTINAL TRACT

The microbial flora of the gastrointestinal tract are much more complex than those of the respiratory tract. In fact, there are close to ten times more bacterial