

## Review Article

# Diagnostic Options for Investigating Viral Respiratory Pathogens in Sudden Unexpected Death in Infancy (SUDI) Cases

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## Abstract

Sudden and unexpected deaths in infants have occurred for centuries. It has generally been referred to as sudden infant death syndrome (SIDS). A new concept, called sudden unexpected death in infancy (SUDI) was introduced in 1989, which is used for all unexpected deaths in infants and babies, usually during sleep, where fatal injury can be excluded. By definition, cases that remain unexplained after thorough investigation are still classified as SIDS.

Many risk factors have been associated with SUDI, e.g. poor socioeconomic conditions and prenatal care, multiple pregnancies, parental drug use and smoking, gender, low birth weight, recent infection and the sleeping environment. Ultimately, SUDI is most probably a result of a combination of predisposing factors, external stresses and underlying vulnerabilities, although the exact mechanism of death remains unknown.

Viral infections are common in infants and have repeatedly been implicated in SUDI. Respiratory infections occur frequently in infancy and early childhood and inflammatory changes in the respiratory tract in SUDI cases is often found.

Different diagnostic approaches for investigating respiratory viruses in SUDI cases have been reported in the literature, but in the absence of standardised SUDI investigation protocols, research from different centres cannot be compared. Viral viability is compromised in post-mortem samples and results should be interpreted with care, as the mere presence of a pathogen does not confirm that to be the cause of death. It is therefore imperative to use a combination of diagnostic approaches in parallel with epidemiological and clinical information in SUDI cases.

## ABBREVIATIONS

IMR: infant mortality rate; SIDS: sudden infant death syndrome; SUDI: sudden unexpected death in infancy; DNA: deoxyribonucleic acid; RNA: ribonucleic acid; CMV: cytomegalovirus; IHC: immunohistochemistry; ISH: *in situ* hybridisation; EM: electron microscopy; IF: immunofluorescence; PCR: polymerase chain reaction.

## INTRODUCTION

The infant mortality rate (IMR) is an estimate of the health status of a country and represents the number of infant deaths for every 1000 live births [1,2]. The four leading causes of infant mortality worldwide are congenital abnormalities, sudden

infant death syndrome (SIDS), prematurity and low birth weight [2]. Despite a drastic decline in the IMR of many regions, SIDS remains one of the most frequent causes of death in infancy [2-6].

SIDS is defined as the sudden and unexpected death of an infant younger than one year, which remains unexplained after a complete autopsy, death scene examination and review of the clinical history [7,8]. Prior to any medico-legal investigation, sudden and unexpected infant deaths are categorised as sudden unexpected death in infancy (SUDI).

Several risk factors have been identified, including socio-demographics, parental smoking and/or substance abuse, insufficient maternal education, multiple pregnancies, low birth

weight, lack of breastfeeding, male gender, recent infections, sleeping position and – environment and genetics, [3,6,9-14]. More SUDI cases also occur during the colder months of the year, suggesting a seasonal aetiology [15-17].

Acute respiratory infections occur often in infants and can sometimes be fatal [18-21]. Young children can have up to eight respiratory infections annually and although this rate is comparable throughout the world, a much worse prognosis is found in areas such as rural Africa, where patients suffer from poor health, malnutrition, high incidences of prematurity, anaemia and other co-morbidities, including malaria and human immune deficiency virus [19,22].

Pulmonary viral infections are the most frequently defined cause of death in SUDI cases worldwide [17,23,24]. Both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses have been detected in these cases, including enterovirus, human adenovirus, Epstein-Barr virus, parvovirus B19, human herpes simplex virus, cytomegalovirus (CMV), human rhinovirus, influenza virus, respiratory syncytial virus, human parainfluenza virus and rotavirus [14,15,25-27]. However, similar viruses often cause mild illness or self-limiting infections in living babies and infants in the general population [28,29]. Positive results should therefore be interpreted with caution and correlated with micro- and macroscopic autopsy findings [18].

## LABORATORY INVESTIGATIONS

Different diagnostic approaches for investigating viral respiratory pathogens in SUDI cases have been reported in the literature, such as histology, cell cultures, immunohistochemistry (IHC), *in-situ* hybridisation (ISH), electron microscopy (EM), immunofluorescence (IF), quantitative and qualitative real-time polymerase chain reaction (PCR) [25,27,30,31]. The latest technology is luminex, which have similar or superior sensitivity compared to direct IF, culture and PCR methods [32].

Obtaining viable lung tissue from SUDI cases for diagnostic purposes has proven to be challenging. Low viral load or extended post-mortem intervals may negatively affect virus viability and nucleic acid integrity, resulting in reduced viral detection [33]. Routine samples are usually formalin-fixed after the autopsy, which can also compromise the quality and integrity of nucleic acids present in the tissue [27]. Autolysis and putrefaction start shortly after death and can lead to overgrowth of post-mortem organisms, such as *α-haemolytic streptococci*, *Staphylococcus epidermidis* and *Escherichia coli*, seriously hampering viral detection [34].

The key qualities required for any method used for viral detection are sensitivity and specificity or selectivity. Sensitivity represents the limit of detection of the assay and is particularly important in early detection to aid in conclusive diagnosis and patient management. Specificity or selectivity refers to the measure of accurate determination of a particular virus in the presence of other target organisms [35]. The difference between specificity and selectivity is that a specific reaction will only occur with the substance of interest, while a selective reaction can occur with other substances, but shows a degree of preference for the substance of interest [36].

## Histology

Standard haematoxylin and eosin stained sections are provide information about trafficking and localisation of inflammatory cells, as well as the extent of necrosis and apoptosis [37]. The cell nucleus typically stains with haematoxylin, while the cytoplasm absorbs the eosin. Although it is possible to visualise significant changes in the respiratory tract due to infection [38], it is not possible to distinguish between different viruses [18].

The most important histological finding in SUDI cases is interstitial pneumonitis, which can be classified as Grade 1 or very mild (barely present, often focal, and with no overall alteration of the alveolar septa), Grade 2 or mild (relatively diffuse and mild thickening of alveolar septa), Grade 3 or moderate (diffuse involvement and associated with interstitial oedema) or Grade 4 or severe (diffuse infiltration associated with significantly widened alveolar septa) [39].

Histological changes can include oedema, congestion, focal collapse, alveolar debris or haemorrhage, bronchiolitis, bronchopneumonia and pneumonitis. These changes, although suggestive of a viral infection, may also be non-specific and should be supplemented with additional diagnostic tests [18,30].

## Cell culture methods

The use of cultured eukaryotic cells in a controlled environment for propagation and isolation of different viruses has been used since the 1960s and is still considered the gold standard for the detection of respiratory viruses [14,28,35,40,41]. Most of the emerging techniques for viral detection are validated against the cell culture technique. Primary (human fibroblast, Rhesus monkey kidney, etc) and continuous cell lines (human lung carcinoma, etc) have the ability to support the replication of a wide variety of clinically relevant viruses. Specimens are inoculated onto these cell monolayers and then monitored by light microscopy for cytopathic effect, which is indicative of viral growth or a positive culture. The presence of a specific virus must then be confirmed by immunofluorescent staining using virus-specific, fluorescently-labelled antibodies. This method is able to demonstrate the viability of a virus, but can take eight to ten days for sufficient viral growth, which limits the diagnostic value of such methods.

Modified, centrifugation-enhanced shell-vial cultures can however, greatly shorten the incubation period necessary for successful culture. A cell monolayer is grown on a coverslip in a small vial into which the test specimen is inoculated and incubated. This method is quicker and cheaper than conventional cell cultures, but generally still as sensitive [42]. However, in selected cases such as CMV, it is less sensitive than conventional cell cultures [43,44].

The use of mixed cell lines, such as a combination of Hep-2 and Madin-Darby Canine Kidney cell lines, can further contribute to a shorter turn-around-time [41,45].

The biggest limitation in using this method for investigation of respiratory viruses in SUDI cases is the time delay between death and post mortem examination, where degradation of possible viral particles can render the virus unable to replicate, resulting in false negative results.

## Immunohistochemistry

This method utilises a monoclonal or polyclonal antibody that binds with high affinity to its corresponding antigen. It is then incubated with a conjugated secondary antibody that will bind to the Fc portion of the primary antibody and when the chromogen is added, it will deposit at sites where the primary antibody is bound and can be visualised under a light microscope. The diagnosis is based on the distribution of inclusion bodies in tissue and cells, tissue structure and presence of inflammation and other structural changes [26,37,46].

IHC can be used on fresh and unfixed tissue, but formalin-fixed, paraffin-embedded tissue is most commonly used. However, fixation masks the antigenicity of the tissue by reversible cross-linkages of protein amino acid residues by methylene bridges, rendering the antigen invisible and necessitating an antigen retrieval step. The amount of cross-linking can be controlled by the fixation time, pH or concentration of the fixative. The antigen retrieval step breaks these cross-linkages and can be done with heat (heat-induced epitope retrieval) and enzyme-based methods, radiation or boiling the tissue sections [47-49].

IHC is an expensive method and even though it can confirm the presence of specific antigen in the tissue, it cannot quantify the infection [50]. False negative results occur frequently, possibly due to subjective interpretation. Differentiation between background staining and true positive results is often difficult [51].

## In situ hybridisation

ISH uses a non-radioactive RNA probe to detect mRNA in paraffin-embedded tissue sections. A digoxenin-labelled probe is used to hybridise specific sequences of single-stranded cell- and tissue-bound RNA and DNA with single-stranded labelled probes of complementary sequences. Because every infectious organism has unique segments of DNA or RNA that are not found in other organisms, cells, or tissues, this method can localise single-copy genes and mRNA transcripts in samples with very low number of cells [52].

Both IHC and ISH hybridisation use paraffin-embedded tissue to demonstrate the presence of specific organisms. IHC is the method of choice in active infections where active viral replication is present, whereas ISH is more suitable in cases of latent infections, such as CMV and human herpesviruses. Both methods are useful in epidemiological studies, as the paraffin-embedded tissue maintain their structural characteristics for long periods of time. IHC is relatively quick and inexpensive as opposed to other techniques. The main disadvantages are the difficulty to distinguish between background and specific staining and despite high sensitivity and specificity, non-specific binding to tissue components such as neurons, glandular epithelium and collagen can occur [52].

## Electron microscopy

Morphological differences between viruses are visible under an electron microscope and a number of new viruses have been described with the aid of EM, including Hendra and Nipah (Henipa) viruses. It does not need any target specific reagents,

but instead allows pathogens to be distinguished on morphology and ultra structure. It is possible to view the presence of all viruses, provided that the viral load is high enough. Due to a low sensitivity, it needs approximately 1 million viral particles per millilitre of fluid for a positive result and low positives often are regarded as negative. This is the main reason that EM has limited diagnostic applications [53,54].

EM has been used in the investigation of SUDI cases to successfully detect viral infections, although at a much lower rate than other diagnostic tests [14,30]. Autolysis of cells starts shortly after death and subsequently results in post-mortem tissue that is not always suitable for EM purposes [30].

## Immunofluorescence

IF has often been used in SUDI investigations [14,28,55]. It is a cytochemical or histochemical assay that is used to detect antigen on the surface of intact cells, inside cells or in serum or plasma. An antibody is coupled to a fluorescent dye and when the antibody binds to an antigen, fluorescence can be seen under a fluorescent microscope [56].

Direct IF involves the overlay of fluorescein-conjugated antibodies against immunoglobulins (IgG, IgM and IgA) and complement and is done on tissue sections. Indirect IF uses an unlabelled primary antibody which is specific for the antigen, but then needs a secondary, fluorescently labelled, antibody specific for the primary antibody [56,57].

Direct IF is a faster assay than indirect IF, but often produces a lower signal. Indirect IF is more sensitive than direct IF, because more than one secondary antibody can bind to each primary antibody, which will strengthen the fluorescent signal, but includes the potential for cross-reactivity [57].

Direct fluorescent antibody tests can be performed in an hour and can detect approximately eight of the common respiratory viruses. The specificity of this test is high, but the sensitivity depends on the specific virus or strain and can be as low as 50% in the case of Adenovirus [58].

## Conventional and Real-time PCR

PCR or nucleic acid amplification tests have superior diagnostic sensitivity and specificity for detection of respiratory viruses compared to viral culture techniques and are gaining ground to become the new, more sensitive gold standard [58]. It uses the principle of amplifying target DNA or RNA and detecting the product by means of gel-electrophoresis at the end of the assay or a fluorescent dye, which is cleaved off when the probe binds to the target sequence (conventional PCR). This omits a signal after being cleaved and can be visualised in real-time (real-time PCR). Real-time PCR methods allow single step amplification and analysis of viral targets and although multiplex PCR methods can detect larger range of viruses in a single reaction, it requires an additional step for post-amplification detection [32,59,60].

These methods are useful in detecting highly pathogenic organisms, as well as viruses that do not proliferate in normal cell cultures, such as Hepatitis B and C, and parvovirus B-19. It is also a much faster method than conventional culture methods [14,22,25,26,41,61].

PCR tests are very expensive and laborious and are limited to the amount of fluorophores that can successfully be differentiated [32,59]. It is also not able to differentiate between active and latent viral infections, unless a quantitative viral load test is performed [41]. Because of the latent period and periodic reactivation of human herpes viruses such as CMV, a positive PCR result might be the result of a primary infection, a latent virus or a reactivated viral infection [62]. For some other viruses, such as the human rhinovirus, prolonged shedding can last up to three weeks after the initial infection [63,64]. It is thus imperative to interpret positive results together with the clinical history of the patient and evaluation of viral specific immune responses [27,30,65,66].

Although formalin-fixed samples have been used to detect viruses with PCR, significant degradation of RNA and DNA occurs in formalin-fixed cells compared to fresh cells and cells fixed in formalin-free media. Formalin fixation can thus cause fragmentation of RNA and DNA, reducing the nucleic acids suitable for molecular amplification and fresh tissue should be collected and processed for PCR [67,68].

### Luminex

This is a microsphere immune assay based on a universal bead-array. The beads are carboxylated paramagnetic polystyrene, 6.5 µm in diameter and are stained internally with spectrally distinct fluorochromes. Covalent coupling with either proteins, peptides, antibodies, polysaccharides, lipids or oligonucleotides determines the specific application of this method [69].

The viral application is a qualitative nucleic acid multiplex test and can simultaneously detect and identify multiple DNA and RNA viruses in a single reaction. Primers with proprietary universal tags enable a reverse transcriptase PCR step, after which the amplified product is hybridised to a bead array conjugated to specific probes. Streptavidin-R-phycoerythrin conjugate generates a signal for each bead population and enables identification in combination with the virus-specific bead address [32,69].

Similar to Real-time nucleic acid tests, Luminex assays are also superior in terms of sensitivity and turnaround time compared to viral culture methods and its sensitivity has been shown to be comparable to real-time PCR. This method has the potential to accurately detect a wide range of respiratory viruses in a short time in a single reaction [32,59].

### Other

This is by no means an exhaustive list of diagnostic modalities available for respiratory viruses. Additional technologies for viral detection are being developed and refined continuously and include, but are not limited to:

**Microarrays:** uses hybridisation of a nucleic acid sample (target) to a very large set of oligonucleotide probes, which are attached to a solid support, to determine sequence or to detect variations in a gene sequence or expression or for gene mapping [69].

**ELISA:** forms complexes between viral antigen and antibodies, that requires a conjugated secondary antibody and substrate to

produce a colour change, which can be spectrophotometrically measured at a specific wavelength [69].

**Isothermal methods:** helicase dependent amplification or recombinase polymerase amplification is used to separate the strands of the double-stranded template to enable primer binding, such that amplification can occur without the repeat cycles of denaturation and annealing required for PCR [69].

**Loop-mediated isothermal amplification or LAMP assay:** uses three pairs of primers (internal, external and loop primers) to provide at least six primer binding sites, which accelerate amplification by priming at the loop regions [69].

**Next generation sequencing:** applying massively parallel sequencing approaches with subsequent bioinformatics analysis for viral sequences can lead to routine, as well as generic detection of viruses and other pathogens [69].

**Microarrays:** an array of immobilised oligonucleotides containing the genetic information of the virus of interest is screened against a fluorescently labelled PCR product to identify and quantify specific DNA sequences [35].

## CONCLUSION

SUDI is still the leading cause of death in infants in many developed and developing countries and although infection has been implicated in many studies, the exact cause and mechanisms of death in many cases remain unknown. There is consensus in the literature that the cause of death is probably multifactorial, but the contributing of underlying role of viral and microbial infection cannot be ignored [70].

Detection of viral pathogens in SUDI cases poses unique challenges. In the absence of standardised protocols with regard to the diagnostic tests to be performed or the selection of viruses to be investigated, research from different centres is not comparable. Viral viability is compromised in post-mortem samples and limits the value of quantitative analyses. However, qualitative results should be interpreted with care, as the mere presence of a pathogen does not imply that it caused or contributed to death. It is therefore imperative to use a combination of diagnostic approaches in parallel with epidemiological and clinical information in SUDI cases.

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