**Unrecognized viral infections and chromosomal abnormalities as a cause of fetal death – examination with fluorescence in situ hybridization, immunohistochemistry and polymerase chain reaction**

Bente Ediassen Opsjøn1, Svein Arne Nordbø1,2, Christina Vogt1,3

1Department of Laboratory Medicine, Children’s and Women’s Health, Faculty of Medicine, Norwegian University of Science and Technology (NTNU), Trondheim, Norway

2Department of Medical Microbiology, St. Olavs Hospital, Trondheim, Norway

3Department of Pathology, St. Olavs Hospital, Trondheim, Norway

Running head: Examinations in unexplained fetal death

Corresponding author:

Bente Ediassen Opsjøn

Department of Laboratory Medicine, Children’s and Women’s Health, Faculty of Medicine, Norwegian University of Science and Technology (NTNU)

7491 Trondheim

Norway

Telephone: +47 41664880

E-mail: bente.e.opsjon@ntnu.no

Authors: Bente Ediassen Opsjøn, Svein Arne Nordbø, Christina Vogt

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Summary

**Background**

15-50 % of fetal deaths remain unexplained after post-mortem examination depending on inclusion criteria and classification systems being in use. Our aim was to examine a selection of unexplained fetal deaths in order to investigate whether any common chromosomal aberrations or viral infections were present.

**Material and methods**

Autopsy reports from 351 fetal autopsies performed at the Department of Pathology and Medical Genetics at St.Olavs University Hospital from 2001 through 2010 were reviewed. Of these, 105 fetal deaths were classified as unexplained. 30 cases were further examined with fluorescence in situ hybridization (FISH) to detect chromosomal abnormalities regarding chromosome 13, 18 and 21. They were also examined with immunohistochemistry (IHC) and polymerase chain reaction (PCR) to detect infections with cytomegalovirus, parvovirus B19, herpes simplex virus 1 and 2, enterovirus and parechovirus.

**Results**

In two cases, a possible trisomy 13 mosaicism was found. No viral infections were diagnosed.

**Conclusion**

In our selection of 30 unexplained cases, a possible trisomy 13 mosaicism was found in 2 cases, and no viral infections were found. High degree of maceration and missing placental examination often complicate the investigation of fetal death and extensive ancillary examinations do not necessarily contribute to a more specific diagnosis.

**Key words:** Fetal death, autopsy, viral infections, chromosome aberrations

Corresponding author:

Bente Ediassen Opsjøn

Department of Laboratory Medicine, Children’s and Women’s Health, Faculty of Medicine, Norwegian University of Science and Technology (NTNU)

7491 Trondheim, Norway

E-mail: bente.e.opsjon@ntnu.no

**Introduction**

Despite thorough examinations with both traditional and ancillary methods, some fetal deaths remain unexplained. The percentage of unexplained deaths varies between industrialized and developing countries, and by which classification system that is used, ranging from 15 to 50 % (1-3). In unexplained fetal deaths there are often missing clinical data, the placenta has been unavailable for clinical examination, and the fetus itself may be severely macerated (4, 5).

Karyotypic abnormalities are present in about 6-12 % of stillbirths, but cell cultures for karyotyping are often unsuccessful (6). 7-10 % of cytogenetic aberrations in stillbirths are not recognized at the postmortem examination or autopsy (7). The most common abnormalities are similar to those found in live-borns and include monosomy X (23 %), trisomy 21 (23 %), trisomy 18 (21 %) and trisomy 13 (8 %) (6). In first trimester abortions, however, trisomy 16 is the most common chromosome abnormality (8). Fluorescence in situ hybridization (FISH) on formalin fixed tissue has been successfully used on fetal tissue from both spontaneous abortions and intrauterine fetal deaths (IUFD) (7, 9-11). If frozen tissue is available, polymerase chain reactions (PCR) on selected chromosomes is also possible.

Parvovirus B19 is known to cause fetal death by attacking erythropoietic tissue, causing fetal anemia, non-immune hydrops and fetal death (12). Congenital cytomegalovirus (CMV) infection is a well-known cause of birth defects, and is associated with intrauterine growth restriction, as it can impair placental development and function (13). High prevalence of CMV infection in stillbirths and increased prevalence of fetal thrombotic vasculopathy in CMV infected vs. non-infected stillbirth suggests an association between CMV infection during pregnancy and stillbirth (14). Herpes simplex virus (HSV) is more commonly transmitted during labor or through breast feeding than in utero, but is associated with intrauterine growth restriction, multiorgan disease and death (15). Maternal infection with enteroviruses close to term can cause severe clinical manifestations for the neonate, and case reports suggest they might cause fetal death, albeit rarely (16). PCR has successfully been used on formalin-fixed and paraffin embedded tissue for a long time, and has proven successful on material that has been stored for years (17, 18). Using both PCR and immunohistochemistry (IHC) gives us the opportunity to rapidly check several organs for multiple agents and also detect the virus in its specific location within the tissues.

Our aim was to examine a selection of unexplained fetal deaths in order to investigate whether any common chromosomal aberrations or viral infections could be diagnosed.

**Material and methods**

All fetal autopsies performed at the Department of Pathology and Medical Genetics at St. Olavs University Hospital during the years 2001 through 2010 were reviewed. A standardized autopsy protocol was followed, including routine radiography and photographic documentation in selected cases. The autopsies were complete including microscopic examination of organs. Cases were selected based on the following criteria: Gestational age ≥ 12 weeks, death by either IUFD, spontaneous abortion or induction of labor. Gestational age was recorded according to post-partum growth measurements. Induced abortions due to chromosome abnormalities and/or severe/lethal malformations were excluded, as were cases of IUFD/spontaneous abortion in which severe/lethal malformations and/or chromosome abnormalities were present. 351 cases fulfilled the criteria and were included. All cases were sorted into three groups according to autopsy and placental findings: 1. non-infectious placental cause, 2. infectious cause, and 3. inconclusive findings. In this last group clinical information and autopsy findings were insufficient to explain the fetal demise. In some cases the fetus was too macerated or autolyzed to be analyzed properly. An experienced pathologist (CV) was consulted when necessary (19).

The “inconclusive” group consisted of 105 cases. 63 cases were excluded due to insufficient material or poor tissue quality, and in 24 of these cases the placenta had not been sent to the pathology lab for examination. Of the remaining 42 cases, 30 cases were selected for further analysis based on tissue quality determined by microscopy. Sections from paraffin embedded tissue from lungs, liver, kidney, thymus and placenta were selected for further examination. Karyotyping (FISH or G-banding) had been performed on some of the cases previously, with varying results. Available results from microbiologic examinations were evaluated in order to determine maternal and/or fetal status for CMV, parvovirus B19, HSV 1 and 2, enterovirus and parechovirus. Different commercial tests (enzyme immunoessays) for antibody detection were used during the observational period. In some cases PCR had already been performed for supplementary examination. In two cases with missing serology status, maternal serum was retrieved from archives and examined. Due to limited resourced for the current project, only samples from thymus, lung and placenta were examined further.

**Chromosome analysis with fluorescence in situ-hybridization**

Slides from thymus were reviewed by two examiners, the student and the pathologist, and the best preserved area on the slides was marked for further examination. This area was sampled from the paraffin blocks, dewaxed and prepared into a single-cell suspension according to the procedure described by Köpf et al. (20). The staining procedure is described in the Histology FISH Accessory Kit, code K5799 (Dako Denmark A/S, Glostrup, Denmark), starting from staining procedure (section B3), following step 1,method B and then step 2,method C and then further as described. All probes were obtained from Abbott (Abbott Molecular Inc., Wiesbaden, Germany). Probes for chromosome 13 and 21 are locus specific (Vysis LSI13 (13q14) Spectrum Green and Vysis LSI21 (21q22) Spectrum Orange), while probes for chromosome 18 are centromere probes (Vysis CEP18 (D18Z1) Spectrum Aqua). The probes were simultaneously hybridized on one slide.

The finished slides were examined with a Nicon Eclipse 90i fluorescence microscope. For each chromosome, 30 cells per case were evaluated. Only cells with clear, distinguishable signals were evaluated. If 80 % or more of the evaluated cells contained two distinct signals, we classified the result as disomy for the evaluated chromosome. Signals for the three different chromosomes were counted using appropriate filters.

**Viral detection using immunohistochemistry (IHC) and polymerase chain reaction (PCR)**

The 30 cases were evaluated to detect infections with CMV, parvovirus B19, HSV 1 and 2, enterovirus and parechovirus. IHC to detect CMV and parvovirus B-19 was performed in all cases, although many had negative results from previously performed PCR. Maternal HSV 1 and 2 status was evaluated based on serology results; in cases of seronegativity, no further PCR was performed. In cases where a reinfection could not be excluded (IgM negative and IgG positive), placental tissue was examined with PCR for HSV 1 and 2. PCR for enterovirus and parechovirus was performed in the cases where this had not been done previously.

**Immunohistochemistry**

Slides from lungs and placenta were selected for IHC with antibodies against CMV and parvovirus B-19. The tissue sections were deparaffinized in TissueClear (Sakura Finetek Europe B.V) and ethanol, and then rehydrated. To enhance staining, a heat-induced epitope-retrieval was performed using Target Retrieval Solution with pH 9 (Dako, K8004, Dako Denmark A/S, Glostrup, Denmark), pre-heated to 85˚C. The slides were then immersed in the solution and heated to 97 ˚C for 20 minutes and then cooled down to 65 ˚C. After cooling down, the slides were washed in 1xDako Wash buffer with pH 7,6 (Dako S3006) for 2 x 5 minutes. The staining process was performed with a Dako Autostainer. The antibodies were obtained from Dako (Dako Denmark A/S, Glostrup, Denmark). For parvovirus B19 detection, the rabbit polyclonal anti-parvovirus B19 (Dako, B0091) antibody was used diluted in a 1:200 ratio in Antibody Diluent, Dako REALtm (Dako, S2022). For CMV detection, the mouse monoclonal anti-cytomegalovirus clones CCH2 + DDG9 (Dako, M0854) antibody were used diluted in a 1:100 ratio in the same diluent as described above. The slides were stained with diaminobenzidine (Dako, K5007), washed in water and counterstained with hematoxylin. Only distinct nuclear staining was scored as positive. The slides were reviewed by a medical student (BEO), and by a pathologist (CV) when necessary. Positive controls from cases with confirmed CMV and parvovirus infection were used as positive controls.

**Polymerase chain reaction**

The autopsy reports were reviewed to check whether the mother and/or the fetus had been tested for the following viruses: CMV, parvovirus B-19, HSV 1 and 2, enterovirus and parechovirus. In three cases, the results from previous testing of the mother excluded the possibility of a current infection with HSV, enterovirus and parechovirus. Of the cases that had not been tested before, 17 were examined for HSV 1 and 2, 22 were examined for enterovirus and 27 were examined for parechovirus.

Four to five 10 µm thick slides from each paraffin block with placental tissue were transferred to a tube and deparaffinised using Xylol. Further tissue lysis was performed by adding the tissue samples to tubes containing 200 µL ATl-buffer and 20 µL Protease K, vortexing the tubes and incubating them at 55 ˚C overnight while shaking. The available DNA and RNA was extracted in a NucliSens easyMAG extractor (BioMérieux) using On-board lysis protocol. All PCR tests were run in a CFX96TM Thermal Cycler (BioRad) running 40 cycles with 3 steps each: a denaturing step at 95 ˚ C for 10 seconds, a primer annealing step at 55 ˚ C for 10 seconds and an extension step at 72 ˚C for 10 seconds. Multiplex real-time PCR was used for detection of HSV 1 and 2, by an in-house PCR, using TaqMan probes targeting the gB gene (21). TaqMan probes targeting the VP1 gene was used for detection of parvovirus B19 (22), and probes targeting a sequence in the DNA polymerase gene was used for detection of CMV (23).. Random hexamer primers (Promega) and Moloney Murine Leukemia reverse transcriptase (Invitrogen by Life technologies) were used for the synthesis of cDNA. Real-time in-house PCR was used for detection of enterovirus (24), and parechovirus using TaqMan probes (25).

**Results**

The 30 cases included for further analysis are shown in Table 1. Mean maternal age was 29.6 years (range 18 to 44 years), and mean gestational age of the fetuses was 28.3 weeks (range 15 to 42 weeks). 14 (46.7 %) of the fetuses were female, and 16 (53.3. %) were male. 23 fetuses died in utero, 4 died during spontaneous abortion and 3 were from terminated pregnancies. One pregnancy was terminated due to fetal hydrops, one due to severe maternal preeclampsia and fetal hydrops, the last one due to five weeks of oligohydramnion after premature prelabor rupture of membranes. Common autopsy findings were aspiration of amniotic fluid (12), petechiae (6), other signs of asphyxia (1), fetal hydrops (4), nuchal edema (2) and cystic hygroma (2). Some cases had more than one finding. In six cases the autopsy did not reveal any specific findings. Main findings from placental examination were unspecific like intervillous fibrin deposition and in some cases small intervillous thrombi and infarctions.

**FISH**

In 15 cases, the hybridization resulted in an insufficient amount of signals for evaluation. In another three, the hybridization of chromosome 13 and 21 failed, but chromosome 18 could be evaluated. In these three cased there was disomy for chromosome 18. In 10 cases all three chromosomes could be evaluated successfully, and disomy for the chromosomes 13, 18 and 21 was found. In the last two cases we found disomy for chromosome 18 and 21, but trisomy 13 mosaicism was suspected as up to 30 % of the evaluated cells had 3 distinct signals for the chromosome 13 probe.

**IHC**

None of the cases showed positive staining for CMV or parvovirus B19 in sections from lung and placenta. Formalin pigment was present in some of the lung sections, but not in the placental sections. Otherwise, unspecific staining was not a problem.

**PCR**

In three cases, the HSV, enterovirus and parechovirus status was already known to be negative based on a previous maternal test result. In the remaining 27 cases, all PCR analyses performed were negative.

**Discussion**

Our analyses of 30 unexplained fetal deaths yielded few results, however, false negatives cannot be excluded due to the poor quality of the material.

**FISH**

A possible trisomy 13 mosaicism was found in two cases. It is difficult to pinpoint the importance of this finding. In general the significance of mosaicisms depends on several factors: the chromosome in question, location of the mosaicism, the prevalence or number of cells affected, and at which stage of development the errors occur (26). Prenatal diagnostics (chorion villous biopsy or amniocentesis) only provide samples from extraembryonal tissues, and may sometimes be of limited use in diagnosing chromosomal abnormalities in the fetus, i.e. mosaicisms (26).

Since fetuses with malformations were excluded, the chance of finding chromosomal abnormalities is reduced, but with mosaicisms the malformations may be inconspicuous and/or confined to single organs, and therefore be difficult to detect in small and autolyzed fetuses (27). We did not include probes for chromosome 16, which could have been useful for the very small fetuses, since trisomy 16 is the most common chromosome abnormality in early abortions (8). Trisomy 16 mosaicism has also been found in older fetuses and liveborns, sometimes only accompanied by minor malformations confined to single organs (28, 29). Therefore, it could be interesting to examine unexplained stillbirths for other chromosomal abnormalities than those we examined in this study.

We found that FISH yielded insufficient signals in half of the analyzed cases, and that the locus specific probes were more difficult to evaluate than the centromere probe, which yielded stronger signals in general. Since specific centromere probes are not available for all chromosomes, it was not possible to obtain stronger signals for the other chromosomes. Another drawback with FISH is reliability, as the results are dependant on the person reviewing the slides and counting signals, thus including the possibility for human error. Also, disomy for a specific probe does not exclude other chromosomal errors. However, in our experience, sending material from fetal autopsies for cell culture in order to do traditional chromosome analysis (G-banding) is often unsuccessful, and it is not possible to do so when the fetus has been formalin-fixed. Thus, FISH has become the most common method in our laboratory. Recently a PCR based method applicable on frozen tissue was introduced in our lab.

While our examinations yielded few results, diagnosing chromosomal aberrations have important consequences for the parents and the clinicians since the recurrence risk is low and prenatal diagnostics are available for subsequent pregnancies (11). However, it is not always possible to determine whether the chromosome aberration was related to the fetal demise or not.

**IHC and PCR**

A Norwegian study showed that 37.2 % and 40.3 % of pregnant women are susceptible to CMV and parvovirus B19, respectively (30), which means that a significant proportion of Norwegian pregnant women are at risk of contracting a primary infection during pregnancy. Even in pre-pregnancy seropositive mothers, secondary CMV infections during the current pregnancy can cause sequelae for the fetus (31). The contribution of viral infections to fetal death has been less investigated than the role of bacterial infections. In studies regarding fetal and neonatal deaths due to infections, 6.5 % of late fetal deaths (GW 20-23) and 14.5 % of stillbirths were attributed to viral causes (32), and viral genomes are detected more often in placental tissue from fetal deaths than in healthy live-borns (33). In preterm prelabor rupture of the membranes (PPROM), however, there is conflicting evidence regarding a viral contribution, and viral genomes are rarely present in these cases (34). Adams et al. found an association between intrauterine growth restriction, nonimmune fetal hydrops, hand/foot anomalies or neural tube defects, and PCR positivity for certain viruses, where adenovirus, CMV and enterovirus were most common (35). In asymptomatic fetuses, PCR positivity does not seem to increase the risk for adverse perinatal outcome (36). None of the 30 cases were found positive for CMV or parvovirus B19 using IHC. Similarly, PCR for HSV 1 and 2, enterovirus and parechovirus were all negative. 23 of our samples were from IUFDs, and many had non-specific autopsy findings similar to those described in the mentioned studies, although our sample size was smaller. PCR is highly sensitive and specific, and false negative results are less common than for other methods (35). Since none of the samples tested by PCR showed inhibition, underreporting in the presence of viral genome, if intact, is unlikely. However, due to the variable quality of the material, we cannot be certain whether any viral genome has been present at some point.

**Strengths and weaknesses**

In about ¼ of the 105 cases the placenta was missing, and these cases were excluded from further analysis. The most macerated cases were also excluded since it was less probable to get valid results in such cases. The degree of maceration was described in general terms like “slight”, “considerable”, and “pronounced” in the autopsy reports, which makes categorization difficult. When evaluating hematoxylin-eosin stained slides, the tissue quality was highly variable. At best, the architecture of the organ was well preserved with only minor nuclear degradation. At worst, only shadow-like remains could be observed. About 1/3 of the best preserved cases with unknown cause of death were further examined. As the tissue quality limits the diagnostic process and since only 1/3 of the inconclusive cases were tested, it is not possible to know whether the tested cases are representative for the whole group. These cases had better preserved tissue and since FISH was successful in only half of the cases, it is not probable that it had been possible to obtain signals in the more macerated cases. We considered Microarray Testing as others have found this useful in stillbirths (37), but the method has not yet been implemented in our lab. As for IHC, results on macerated tissue are unpredictable, with less chance of getting valid results if the more macerated cases had been tested. Since PCR is a very sensitive method, the negative results are probably valid.

**Conclusion**

In our selection of 30 unexplained cases, a possible trisomy 13 mosaicism was found in 2 cases, and no viral infections were found. High degree of maceration and missing placental examination often complicate the investigation of fetal death, and extensive ancillary examinations do not necessarily contribute to a more specific diagnosis.

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**Table 1: Characteristics and test results of the examined unexplained cases of fetal death.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Mat. age (years)** | **GA**  | **Fetal gender** | **Manner of death** | **Reported findings** | **IHC** | **PCR and other\*** | **FISH** |
| 24 | 34 | Male | IUFD | No specific findings | Neg | Neg | Disomy |
| 30 | 42 | Female | IUFD | Pleural petecchia, signs of asphyxia | Neg | Neg | Disomy |
| 32 | 20 | Female | TOP | Fetal hydrops | Neg | Neg | Disomy |
| 30 | 31 | Female | IUFD | No specific findings | Neg | Neg | Insufficient |
| 31 | 15 | Male | IUFD | No specific findings | Neg | Neg | Insufficient |
| 30 | 22 | Female | IUFD | Aspiration of amniotic fluid, signs of asphyxia | Neg | Neg | Plausible trisomy 13 mosaicism |
| 32 | 36 | Female | IUFD | Fetal hydrops | Neg | Neg | Plausible trisomy 13 mosaicism |
| 25 | 16 | Female | SA | Bicornate uterus, nuchal edema, VSD | Neg | Neg | Disomy 18, insufficient for 13 and 21 |
| 39 | 26 | Female | IUFD | Pleural petecchiae | Neg | Neg | Insufficient |
| 32 | 26 | Female | IUFD | Aspiration of amniotic fluid | Neg | Neg | Insufficient |
| 25 | 40 | Male | IUFD | Aspiration of amniotic fluid | Neg | Neg | Insufficient |
| 23 | 40 | Male | IUFD | Aspiration of amniotic fluid | Neg | Neg | Insufficient |
| 18 | 18 | Female | IUFD | Fetal hydrops, cystic hygroma, coarctation of the aorta | Neg | Neg | Disomy |
| 26 | 39 | Male | IUFD | Pleural and thymic petecchiae. amniotic fluid aspiration  | Neg | Neg | Disomy |
| 28 | 42 | Male | IUFD | Pericardial petecchiae, meconium aspiration | Neg | Neg | Insufficient |
| 44 | 37 | Male | IUFD | Aspiration of amniotic fluid | Neg | Neg | Insufficient |
| 33 | 31 | Male | TOP | Fetal hydrops, hypoplastic lungs, thymic stress involution | Neg | Neg | Disomy 18, insufficient for 13 and 21 |
| 30 | 41 | Male | IUFD | Aspiration of amniotic fluid, thymic stress involution | Neg | Neg | Disomy |
| 28 | 25 | Male | SA | Fetal hydrops, cystic hygroma, hydrothorax, hypoplastic lungs, germinal layer hemorrhage  | Neg | Neg | Disomy 18, insufficient for 13 and 21 |
| 22 | 24 | Female | IUFD | No specific findings | Neg | Neg | Insufficient |
| 37 | 22 | Male | SA | Hypoxic hemorrhages in thymus and lungs, slight adrenal cytomegaly. | Neg | Neg | Disomy |
| 33 | 16 | Female | TOP | No specific findings | Neg | Neg | Disomy |
| 20 | 16 | Female | SA | No specific findings | Neg | Neg | Disomy |
| 38 | 40 | Male | IUFD | Pleural and pericardial petecchiae, aspiration of amniotic fluid,  | Neg | Neg | Insufficient |
| 36 | 38 | Male | IUFD | Pleural and pericardial petecchiae, subcapsular liver hemorrhages, nesidioblastosis | Neg | Neg | Disomy |
| 33 | 34 | Male | IUFD | Aspiration of amniotic fluid | Neg | Neg | Insufficient |
| 27 | 35 | Male | IUFD | Pleural petecchiae, aspiration of amniotic fluid, thymic stress involution  | Neg | Neg | Insufficient |
| 38 | 16 | Male | IUFD | Hypercoiled umbilical cord | Neg | Neg | Insufficient |
| 32 | 15 | Female | IUFD | Nuchal edema | Neg | Neg | Insufficient |
| 19 | 41 | Female | IUFD |  Aspiration of amniotic fluid, thymic stress involution | Neg | Neg | Insufficient |

\*Other methods include serology results from maternal testing.