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**Viral Infection in Hydrops Fetalis, Spontaneous Abortion
and Unexplained Fetal Death in utero**

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3 1 Viral Detection in Hydrops Fetalis, Spontaneous Abortion and Unexplained Fetal Death *in*
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51 17 Shortened Title: Virus detection in fetal death.
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3 20 Abstract
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6 21 This study was undertaken to investigate the occurrence of viral infection in fetal death by
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9 22 examining tissues for the presence of DNA of several viral agents.

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12 23 Tissue specimens including heart, kidney, liver, lung and placenta of 73 cases of fetal death
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14 24 were examined with 27 cases of elective termination of pregnancy as a control group. DNA
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17 25 extracted from these samples was tested for the presence of HSV, CMV, EBV, VZV, HHV-6,
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19 26 HHV-7 and PVB19. Viral DNA was found in one or more tissue samples from 25/73 cases
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21 27 (34%): CMV in 20, HSV in 5, parvovirus B19 in 5, HHV-7 in 3 and HHV-6 in 2. The
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23
24 28 presence of HHV-6 in fetal tissue has been reported rarely. No study so far has reported the
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26 29 detection of HHV-7 in fetal tissues with normal or adverse outcomes. Viral DNA was not
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29 30 found in any of the termination of pregnancy samples.

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32 31 Among the positive cases, 8 had dual infection. One further case was positive for three
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34 32 viruses: HSV, CMV and HHV-7. HHV-6 was the sole infectious agent in two cases, HHV-7
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36 33 in one case, PVB19 in three and CMV in 10 cases. The finding of multiple viral DNA in 12%
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39 34 of the cases suggests the involvement of complex risk factors in cases of fetal loss. Although
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41 35 the cause of fetal death often includes other factors (e.g. chromosomal abnormalities) these
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44 36 data suggest the incidence of viral infective aetiology may be higher than considered
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46 37 previously. However, larger studies are required to establish this link.
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52 39 Key Words
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56 40 Herpes viruses; parvovirus B19; polymerase chain reaction.
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3 42 Introduction
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6 43 Non-immune fetal hydrops, spontaneous abortion and unexplained fetal death *in utero*, can
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8 44 follow viral infection (Grose et al., 1989). Surveys of fetal hydrops cases have concluded that
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10 45 viruses are an uncommon cause of the condition and have attributed most cases to
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12 46 cardiovascular problems and/or chromosomal abnormalities (Machin 1989; Boyd & Keeling
13
14 47 1992). However, much of the data used in support of this hypothesis were collected before
15
16 48 the widespread use of molecular biology techniques, particularly the polymerase chain
17
18 49 reaction (PCR), in the diagnosis of virus infection (Weiner 1997). In addition to confirming
19
20 50 the involvement of parvovirus B19 (PVB19), these newer techniques are providing evidence
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22 51 of the presence of other viruses in fetal abnormalities.
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28 52 Other viruses, including cytomegalovirus (CMV) and herpes simplex virus (HSV) have been
29
30 53 described in association with non-immune hydrops and fetal infection (Barron & Pass, 1995).
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32 54 In a study of samples (mainly amniotic fluid) from 303 abnormal pregnancies at risk for viral
33
34 55 infections (van den Veyver et al., 1998), viral genome was detected in 41% of patients, with
35
36 56 adenovirus (24%), CMV (10%) and enteroviruses (7%) being the most common. Virus was
37
38 57 detected in only 3/154 normal controls. Ashshi et al., (2000), reported the detection of human
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40 58 herpes virus 6 (HHV-6) DNA in two of 8 fetuses with fetal hydrops and none of the ten non-
41
42 59 hydropic fetuses. Chromosomal abnormalities were noted in both cases with the positive
43
44 60 HHV-6 DNA. More recently, Syridou et al., (2008) detected viral DNA in 21/62 (34%)
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46 61 placenta tissues of fetal deaths. This evidence suggests viral infection may be more common
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48 62 in fetal death than supposed previously.
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55 63 Many of the previous studies have used amniotic fluid or placental tissue only. In the present
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57 64 study, in addition to placental tissue, fetal heart, kidney, liver and lung tissue has been tested
58
59 65 by PCR to investigate the occurrence in cases of fetal death of the following viral agents:
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3 66 HSV, CMV, Epstein-Barr virus (EBV), varicella zoster virus (VZV), HHV-6, human herpes
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6 67 virus 7 (HHV-7) and PVB19.
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For Peer Review

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6 86 Ethical permission
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10 87 Ethical approval for this study was provided by the Central Manchester Local Research
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12 88 Ethics Committee (REC reference: 05/Q1407/193) for the Manchester specimens and from
13

14 89 the Women's and Children's Hospital Research Ethics Committee in Adelaide (REC
15

16 90 reference: 1099/4/2003 and 1099/4/2006) for the Australian specimens.
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20 91 Specimens
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23 92 A total of 197 tissue specimens of heart, kidney, liver, lung and placenta of 48 cases of fetal
24

25 93 death in 1997 were obtained from the paediatric pathology archive, Department of
26

27 94 Histopathology, Manchester Royal Infirmary, Manchester, UK and 119 tissue specimens of
28

29 95 heart, kidney, liver, lung and placenta of 25 cases of fetal death were obtained from
30

31 96 Department of Histopathology, Women's and Children's Hospital, North Adelaide, Australia.
32

33 97 These cases were selected because autopsy had not established a specific cause of death.
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35 98 Samples were provided as 5×10µm thin sections from formalin-fixed, paraffin-embedded
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37 99 tissues and had been anonymised and encoded. The codes were broken only after all samples
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39 100 had been tested. Sections from 27 cases (all from Adelaide) of elective termination of
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41 101 pregnancy within the first trimester with informed written consent from the next of kin were
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43 102 used as a control group.
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47 103 DNA extraction and polymerase chain reactions
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50 104 Paraffin removal from the tissue sections, DNA extraction with guanidinium thiocyanate,
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52 105 anti-contamination methods and PCR conditions for the amplification of human and viral
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54 106 DNA were described by Cooper et al (2008). Additional PCR were used to confirm the
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56 107 detection of HSV, HHV-6 and PVB19 DNA. Primers YS1
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3 108 (5'GACTACCTGGAGATCGAG3') and YS2 (5'TGAACCCGTACACCGAGT3') amplify a
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6 109 fragment of 211bp of the DNA polymerase gene of the HSV-1 and HSV-2 genomes
7
8 110 (Schlesinger et al. 1995). Primers SY3 (5'AGTGACAGATCTGGGCGGCCTAATAACTT3')
9
10 111 and SY4 (5' AGGTGCTGAGTGATCAGTTTCATAACCAA3') amplify a 164bp fragment
11
12 112 of the IE1 gene (U89) in HHV-6 type A and a 420bp fragment of the same IE1 gene
13
14 113 designated as U90 in HHV-6 type B (Yalcin et al, 1994). For PVB19, Prm4a
15
16 114 (5'AACGCCTCAGAAAATACCC3') and Prm4b (5'TAAGTGCTGAAACTCTAAAGG3')
17
18 115 as external primers, and B19-1 (5'CAAAAGCATGTGGAGTGAGG3') and B19-2
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20 116 (5'ACCTTATAATGGTGCTCTGGG3') as internal primers were used in a nested PCR to
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22 117 amplify a 104bp fragment of the VP1 region (Musiani et al. 1993; Umene & Nunoue 1995).

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28 118 Reaction mixtures for these additional PCR contained 10mM Tris/HCl pH8.3, 50mM KCl,
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30 119 1.5mM MgCl₂, 0.001% w/v gelatin, 0.2mM dNTPs, 0.2μM of each primer (0.5μM for
31
32 120 PVB19), 1.25 units of Amplitaq DNA polymerase (Amplitaq Gold for HHV-6) and 5μl of
33
34 121 sample DNA in a total volume of 50μl. The cycling conditions were 94°C/7min (94°C/12min
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36 122 for HHV-6) (1 cycle); 94°C/30s, 55°C/30s, 72°C/30s (72°C/45s for HSV) (40 cycles);
37
38 123 72°C/2min (1 cycle).

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43 124 The detection limits/assay of the PCRs for the various agents were 1-10 copies for HHV-6
44
45 125 and PVB19, 25 copies for CMV and HSV, 60 copies for EBV and VZV and 1000 copies for
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47 126 HHV-7. All were determined with serial dilutions of control DNA of known copy number
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49 127 and were repeated whenever a new batch of any PCR reagent was obtained.

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3 131 Results.

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6 132 Detection of viral DNA

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10 133 All 316 tissue samples from the 73 cases of fetal death and all the 27 tissue samples from the

11 134 cases of termination of pregnancy used as controls yielded human GAPDH gene product

12 135 following amplification, suggesting successful DNA extraction and no non-specific inhibition

13 136 of the PCRs.

14 137 Viral DNA was found in one or more tissue samples from a total of 25/73 cases (34%): CMV

15 138 in 20 (27.4%), HSV in 5 (6.8%), PVB19 in 5 (6.8%), HHV-7 in 3 (4.1%) and HHV-6 in 2

16 139 (2.7%). Among the positive cases, 8 had dual infection, all of which were positive for CMV

17 140 with either PVB19 (3 cases), HSV (4 cases) or HHV-7 (1 case). One further case was positive

18 141 for three viruses: HSV, CMV and HHV-7. HHV-6 was the sole infectious agent in 2 cases,

19 142 HHV-7 in 1 case, PVB19 in 2 cases and CMV in 10 cases. Neither EBV nor VZV were

20 143 detected in any of the fetal death samples. None of the viruses tested were detected in any of

21 144 the 27 elective terminations of pregnancy controls.

22 145 Alternative primers were used for confirmation of the positive cases for three of the viruses,

23 146 PVB19, HHV-6 and HSV. All five PVB19 positive cases remained positive using

24 147 Prm4a/Prm4b and B19-1/B19-2 nested primers that amplify a portion of the VP1 gene

25 148 despite being 10-fold less sensitive than the P1/P6 and P2/P5 nested primers that amplify a

26 149 portion of the NS1 gene. Both HHV-6 cases were confirmed with the alternative primers

27 150 SY1/SY2 that amplify part of the IE1 gene and have the same sensitivity as the H6-6/H6-7

28 151 primers that amplify a portion of the U67 gene. The former primers generate a product of

29 152 194bp for variant A and 420bp for variant B. From this it was clear that case H4 was infected

30 153 with HHV-6 type A and case F36 with HHV-6 type B. The alternative primers (YS1/YS2)

31 154 that amplify a portion of the DNA polymerase gene of HSV could only confirm 3/5 cases

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3 155 positive by the KS30/KS31 primers that amplify a portion of the glycoprotein B gene. This
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6 156 may be because the former primers are 10-fold less sensitive. However, YS1/YS2 allowed
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8 157 typing of HSV using *HinfI* and *BamH1* restriction endonucleases. HSV1 DNA amplified by
9
10 158 YS1/YS2 is cut by *HinfI* but not *BamH1* whereas HSV2 is the reverse. This showed that two
11
12 159 cases (S11, F11) were infected with HSV1 and the third case (F38) with HSV2.

160 Hydrops fetalis

161 A total of 13 hydrops fetalis cases were tested, 11 from Manchester and 2 from Adelaide;
162 only 3/13 were males and the gestational age ranged from 15-20 weeks (Table I). Three (H4,
163 H5, H8) had chromosomal abnormalities and a further 3 had cardiovascular problems (H1,
164 H2, H11), conditions that are often associated with fetal hydrops. Viral infection was found
165 in 5/13 cases (38.46%), 1 male and 4 females with the lung being positive for virus DNA in
166 all of the 5 positive cases, the liver in 4 cases and the heart and kidney in 3 cases. Two cases
167 were positive for CMV, two had a dual infection of CMV and PVB19 and the other was
168 positive for HHV-6 alone.

169 Spontaneous abortion

170 The 32 cases of spontaneous abortion (9 from Adelaide) had a gestation age from 13-19
171 weeks (Table II). There were 21 males and 11 females. Six cases were reported to have
172 chorioamnionitis (F3, F13, S2, S3, S7, S12), although the mixed bacterial growth detected in
173 some of these cases was not thought to be the cause. Fourteen further cases were stated to
174 have an unknown cause of death. Viral infection was detected in 10/32 cases (31.25%) and
175 appeared to be more common in females (5/11; 45.45%) than males (5/21; 23.80%) but the
176 difference is not statistically significant ($p=0.25$) by Fisher's two-tailed exact test. CMV
177 DNA was detected in all 10 positive cases, 3 as a dual infection with HSV, 1 as a dual

178 Table I Autopsy findings among hydrops fetalis cases.

179

Code	Sex	Gestation (wks)	Organs	Autopsy data	Viral PCR
H1	F	18	Li, L, P	Pulmonary & bilateral renal medullary hypoplasia	Neg
H2	M	18	H, K, Li, L, P	Cardiovascular defects, pulmonary hypoplasia, hepatic necrosis	Neg
H3	F	19	H, K, Li, L, P	Cause unknown	Neg
H4	F	17	H, K, Li, L	Down's syndrome	HHV-6 type A
H5	F	20	K, Li, L, P	Turner's syndrome	Neg
H7	F	17	H, K, Li, L	Cystic hygroma & unilateral hydronephrosis	Neg
H8	F	19	K, Li, L	Down's syndrome	Neg
H11	M	20	H, K, Li, L, P	Cardiovascular defects	CMV, PVB19
H12	F	20	H, K, Li, L, P	Dandy-Walker syndrome	CMV, PVB19
H13	F	16	H, K, Li, L	Cystic hygroma	CMV
H14	M	19	H, K, Li, L, P	Cause unknown	Neg
H15*	F	15	H, K, Li, L, P	Intrauterine fetal death, cystic hygroma, cause unknown	Neg
H16*	F	17	H, K, Li, L, P	Intrauterine fetal death, cause unknown	CMV

*Cases from Adelaide;
Organs in bold are positive for viral DNA;
H=heart,
K=kidney,
Li=liver,
L=lung,
P=placenta.

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200 Table II Autopsy findings among spontaneous abortion cases.

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	Code	Sex	Gestation (wks)	Organs	Autopsy data Journal of Medical Virology	Viral PCR
1						
2	F3	M	19	H, K, Li, L, P	Chorioamnionitis, cervical incompetence	Neg
3						
4	F6	M	16	H, L, P	Omphalocele	Neg
5						
6	F7	F	17	P	Advanced maceration	Neg
7						
8						
9	F9	F	14	H, K, Li, L	Left symmetry syndrome	Neg
10						
11	F10	M	17	L, P	Maceration	Neg
12						
13	F11	F	16	H, K, Li, L, P	Placental haemorrhage	CMV, HHV-7, HSV1
14						
15	F13	F	17	H, K, Li, L, P	Chorioamnionitis, premature rupture of membranes, oligohydramnios	CMV, HHV-7
16						
17	F14	F	19	H, K, Li, L, P	Trisomy 18	Neg
18						
19	F18	F	19	H, K, Li	Cause unknown	Neg
20						
21	F19	M	13	H, K, Li, L, P	Monochorionic twin of F20	Neg
22						
23	F20	M	13	H, K, Li, L, P	Monochorionic twin of F19	CMV
24						
25						
26						
27						
28	S2	M	15	H, K, Li, L, P	Chorioamnionitis	Neg
29						
30	S3	M	19	H, K, Li, L, P	Chorioamnionitis	Neg
31						
32	S7	F	19	K, Li, L, P	Chorioamnionitis, intrauterine pneumonia, funiculitis	Neg
33						
34	S8	M	15	L	Cause unknown	Neg
35						
36						
37						
38	S9	M	18	H, K, Li, L	Cause unknown	Neg
39						
40	S10	M	18	H, K, Li, L, P	Twin 2, twin 1 aborted at 9 wks	Neg
41						
42						
43	S11	M	16	H, K, L, P	No malformations, cause unknown	CMV, HSV1
44						
45	S12	F	18	H, K, Li, L, P	Chorioamnionitis, pneumonia	CMV
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*Cases from Adelaide; Organs in bold are positive for viral DNA; H=heart, K=kidney, Li=liver, L=lung, P=placenta.

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3 209 infection with HHV-7 and 1 in combination with both HSV and HHV-7. Viral DNA was
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6 210 detected in most of the available organs apart from F20 where only the kidney was positive
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8 211 and F42 where heart and liver were positive.
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11 212 Intrauterine fetal death
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14 213 There were 28 cases of intrauterine fetal death (14 from Adelaide) with a gestation age range
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17 214 of 20-41 weeks (Table III). Sixteen were male and 12 were female. A diverse range of
18
19 215 conditions was reported at autopsy, including 5 cases of chorioamnionitis (S1, S6, S14, S15,
20
21 216 S19), 3 with chronic villitis (F25, F33, F36) and 4 cases with a small placenta (F25, F32, F33,
22
23 217 F36). Viral DNA was detected in 10/28 cases (35.71%) with 5/12 in females (41.67%) and
24
25 218 5/16 in males (31.25%). CMV DNA was detected in 6 cases, 1 as a dual infection with HSV
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27 219 and 1 as a dual infection with PVB19. Two further cases were positive for PVB19 alone, 1
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29 220 for HHV-6 and 1 for HHV-7. Again, in most cases the virus infection was widespread with
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31 221 all available organs positive for viral DNA in 5 cases.
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228 Table III Autopsy findings among intrauterine fetal death cases.

Code	Sex	Gestation (wks)	Organs	Autopsy data	Viral PCR
F1	M	20	H, K, Li, L, P	Intrauterine growth restriction	Neg
F2	M	21	H, K, Li, L	Amniocentesis	Neg
F4	M	21	K, L, P	Twin to twin transfusion syndrome, twin of F5	Neg
F5	M	21	H, K, Li, L, P	Twin to twin transfusion syndrome, twin of F4	Neg
F8	F	20	K, Li, L, P	Fetal akinesia syndrome	Neg
F12	F	24	K, L, P	Omphalocele	HHV-7
S1	F	20	K, Li, L, P	Acute chorioamnionitis, intrauterine pneumonia	Neg
S4	F	23	H, K, Li, L, P	Hydrocephalus	Neg
S5	M	21	K, Li, L	Cause unknown, mother had fibroids	Neg
S6	M	23	K, Li, L, P	Acute chorioamnionitis	Neg
S13	M	23	K, Li, L	Acute intrauterine hypoxia of unknown cause	CMV, HSV
S14	F	21	H, K, Li, L	Chorioamnionitis, monochorionic twin of S15	CMV

S15	F	21	H, K, Li, L	Chorioamnionitis, monochorionic twin of S14	CMV
S19	F	22	H, K, Li, L, P	Severe chorioamnionitis, pneumonia	Neg
F21*	M	36	H, K, Li, L, P	Cause unknown	Neg
F25*	F	41	H, K, Li, L, P	Cause unknown, small placenta, chronic villitis	Neg
F27*	M	32	H, K, Li, L, P	Maternal atypical pneumonia, diabetes, DIC	Neg
F28*	M	27	H, K, Li, L, P	Intrauterine growth restriction	Neg
F29*	F	40	H, K, Li, L	Cause unknown	Neg
F31*	F	25	H, K, Li, L, P	Cause unknown	Neg
F32*	M	25	H, K, Li, L, P	Placental insufficiency, intrauterine growth restriction	CMV
F33*	M	36	H, K, Li, L, P	Small placenta, chronic villitis, intrauterine growth restriction	PVB19
F34*	M	34	H, K, Li, L, P	Cause unknown	Neg
F35*	F	22	H, K, Li, L, P	PVB19 infection positive by IHC	PVB19
F36*	M	41	H, K, Li, L, P	Small placenta, chronic villitis, intrauterine growth restriction	HHV-6 type B
F39*	M	23	H, K, Li, L, P	Cause unknown, oedematous cord	Neg
F40*	M	36	H, K, Li, L, P	Cause unknown	CMV

S20*	F	21	H, K, Li, L, P	Placental abruption	CMV, PVB19
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230 *Cases from Adelaide; Organs in bold are positive for viral DNA; H=heart, K=kidney, Li=liver, L=lung, P=placenta.

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3 235 Discussion.
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6 236 The specimens in this study were taken from cases of fetal death where a specific cause had
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8 237 not been established. Thus, cases where viral or bacterial infection had been reported as the
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10 238 cause of fetal loss were excluded from the study. The one exception to this was case F35
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12 239 from Adelaide where PVB19 infection was identified previously by immunohistochemistry
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14 240 and had been deliberately included in the encoded samples as known positive control
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16 241 material. Consequently, comparison with detection rates from previous studies requires
17
18 242 caution. Nevertheless, the rate of CMV detection was higher than several previous reports. In
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20 243 this study using fetal organs as well as placenta, the overall detection rate for CMV was
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22 244 27.4% compared with the 10% reported by van der Veyver et al (1998), 5% by Reddy et al
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24 245 (2005) and 16% detected by Syridou et al (2008). This difference may be due to these authors
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26 246 using mainly amniotic fluid or placental tissue where virus presence may be transitory.
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28 247 However, PVB19 was detected in 6.8% of cases in the fetal organs which is lower than the
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30 248 15% rate reported by Tolfvenstam et al (2001) also in fetal organs but comparable to the 5%
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32 249 rate detected by Reddy et al (2005) in amniotic fluid. This difference may be because we
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34 250 have excluded cases already established as PVB19-positive. HSV was detected in 6.8% of
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36 251 cases and is comparable to the 6% detected by van der Veyver et al (1998) and 5% detected
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38 252 by Reddy et al (2005).
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47 253 Three cases of HHV-7 (4.1%) and two cases of HHV-6 (2.7%) were detected. Very little
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49 254 work has been done on these viruses in fetal tissue. HHV-6 was detected in 1.6% (Adams et
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51 255 al 1998) and 1% (Hall et al 2004) of cord blood samples from normal births. Aubin et al
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53 256 (1992) found HHV-6 DNA in several organs from 1/52 fetuses from induced abortions from
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55 257 HIV seropositive women but no obvious abnormality was found. However, Ashshi et al
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57 258 (2000) reported two fetuses (one with Down's syndrome and the other with Turner's
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3 259 syndrome) that were both positive for HHV-6 type A. As HHV-6 can integrate occasionally
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5 260 into the host cell chromosome (Luppi et al 1993; Torelli et al 1995; Daibata et al 1998) it is
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8 261 possible this was the case in the current study. Further exploration of this would require cells
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10 262 to be stimulated into division for chromosomal spreads and with fixed tissue this was clearly
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12 263 not possible. Hall et al (2004) failed to detect HHV-7 DNA in any of the 2129 cord blood
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14 264 samples tested. Chow et al (2006) detected HHV-7 DNA in 1/11 placental tissues from
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17 265 women at high risk of congenital infection. The outcome of the pregnancy was normal and no
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19 266 fetal complications were noted. Viral infection of the placenta indicates risk of vertical
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21 267 transmission but this does not always occur. In this study, HHV-7 DNA was evident in at
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23 268 least two of the fetal organs in all three positive cases and this is the first direct evidence of
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26 269 HHV-7 infection of fetal tissue.

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30 270 Of the 25 fetal death cases positive for virus DNA, there were 8 dual infections and one triple
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32 271 infection. Van der Veyver et al (1998) also reported multiple virus infections in 13/124
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34 272 abnormal pregnancies with 9/13 resulting in fetal death. This suggests multiple virus
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36 273 infections indicate a poor prognosis but further work with larger numbers of patients are
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38 274 required to establish this point.

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42 275 Symptomatic congenital infection in fetuses is more likely after primary maternal infection
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44 276 with CMV (Fowler et al 1992) or HSV (Brown et al 2003) but has also been reported after
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46 277 recurrent maternal infection (Boppana et al 1999; Brown et al 2003). In contrast, the role of
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48 278 CMV (Kost et al 2007) and HSV (Nahmias et al 1971) in fetal death is less well understood.
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50 279 We have no data on the status of the mothers of the positive cases with regard to primary or
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52 280 recurrent infection with either HSV or CMV. The cases with HHV-6 or HHV-7 are more
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54 281 likely to be recurrence as by the age of 8 years more than 90% of children have been infected
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56 282 with both viruses (Tanaka-Taya et al 1996). Fetal death caused by PVB19 is regarded as a

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3 283 consequence of primary maternal infection as maternal immunity completely protects the
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6 284 fetus (Berns & Parrish 2007) although it is interesting to note that this virus often persists in
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8 285 tissues for many months, including in some individuals who are antibody negative (Corcioli
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10 286 et al 2008). Most of the abnormalities reported in the cases positive for viral DNA (e.g.
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12 287 hydrops fetalis, chromosomal and cardiovascular abnormalities) are regarded as risk factors
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15 288 for fetal death and virus infection resulting in further damage may be an additional
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17 289 contributing factor leading to this outcome. However, many of the virus-positive cases had no
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20 290 other obvious problems and here the virus alone may be the cause of death. The viruses
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22 291 detected in this study are widespread and common in the human population and when
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24 292 pregnant women are infected the fetus is usually unaffected. This makes it very difficult to
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27 293 establish a causal effect and we have not done so here. However, these findings suggest virus
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29 294 infection of the fetus is more common than thought previously and more extensive studies of
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31 295 greater numbers of fetal death cases together with data from affected mothers are warranted.
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