Human Cytomegalovirus Infection Is Detected Frequently in Stillbirths and Is Associated With Fetal Thrombotic Vasculopathy

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(See the editorial commentary by Pereira, on pages 1510–2.)

Background. Human cytomegalovirus (CMV) is the most common congenital infection in developed countries and is a known cause of intrauterine fetal death. We examined CMV infection in stillbirths and the relationship with histopathological findings at autopsy.

Methods. We collected liver, kidney, and placenta specimens from 130 stillbirths. CMV DNA and protein were detected using polymerase chain reaction and immunohistochemistry, along with routine autopsy of stillborn infants.

Results. Overall, CMV DNA was detected in 15% of singleton, >20-week stillborn infants. CMV DNA was detected in kidney (9%), liver (11%), and placenta (5%) specimens, with 75% of infections confirmed by immunohistochemistry. Fetal thrombotic vasculopathy was the only histopathological abnormality associated with CMV infection (in 60% CMV-infected vs 28% uninfected stillbirths P = .010).

Conclusions. Stillbirth has multiple etiologies. However, the detection of CMV DNA in 15% of fetal tissues or placentae suggests a strong association between CMV infection in pregnancy and stillbirth. Molecular testing during postmortem investigation has an important role to determine the contribution of CMV infection.
is uncertain without additional supporting evidence of cytopathology, particularly because viral pathogens may also be found in pregnancies with normal outcomes [9]. Intrauterine transmission of cytomegalovirus (CMV) is the leading viral cause of congenital infection in many developed countries [10] and is a known cause of intrauterine death [11, 12]. Maternal primary CMV infection occurs in 0.15%–2% of pregnancies, with 32% of mothers vertically transmitting the virus to the fetus [13]. The mean incidence of CMV is 0.64% of all fetuses and/or live births [13], although CMV infection during pregnancy can also be restricted to the placenta with no further involvement of the fetus [14].

Nucleic acid tests, such as polymerase chain reaction (PCR), are more sensitive and specific for identifying infection [15]. We investigated the etiological role of CMV in stillbirths by combining PCR and immunohistochemistry examination of placenta and fetal tissue (kidney and liver) specimens from a retrospective case series of 130 stillborn infants from a tertiary referral pathology department. We demonstrate abnormalities in histopathology with CMV infection in significant numbers of stillbirths.

**MATERIALS AND METHODS**

**Sample Size and Inclusion Criteria**

During the period January 2005 through December 2006, 442 records were examined from autopsies performed at the Department of Histopathology of the Children’s Hospital at Westmead, a large pediatric anatomical pathology referral center in Sydney, New South Wales, Australia. All autopsies were performed by 2 experienced pediatric histopathologists who were blinded to the results of molecular testing. The autopsy procedures were routine and based on published standard procedures [16, 17]. The cases (n = 130) fitting selection criteria were singleton stillbirths with formalin-fixed, paraffin-embedded kidney, liver, and placental tissue available. Additional selection criteria were ≥20 weeks gestation, no diagnosed cause of death, and having undergone routine histopathological study, including staining for infectious agents if pathologists regarded this as necessary clinically. All cases had bacterial culture of samples of fetal tissues, fetal blood, gastric aspirates, and contents determined at the time of autopsy if fluid was present. Approval for this study was obtained from Human Research Ethics Committee (04-210 [11 August 2005] and 09-012 [19 June 2009]), and site-specific approval was provided by the Children’s Hospital at Westmead (MR 2009-04-03 [27 February 2009]) and the Prince of Wales Hospital (09-G-019 [21 July 2009]) in Sydney, Australia.

**Postmortem Specimens**

Postmortem kidney, liver, and placenta specimens were fixed in formalin prior to embedding in paraffin. Postmortem tissues were examined as part of the routine diagnostic evaluation of stillbirths undergoing autopsy examination. A report containing results of infection screening was issued after completion of standard autopsy tests.

**Molecular Assays**

Extraction of total nucleic acid was performed from three 10-μm sections of paraffin tissue blocks produced during the autopsy. Deparaffinisation, external lysis, and proteinase K digestion were performed before extraction using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche) in accordance with the manufacturer’s recommended method. In brief, deparaffination consisted of 2 rounds of incubation of the tissue sections with 800 μL of xylene for 5 min and centrifugation at 8000g. The tissue pellet was then washed 3 times with 500 μL of 96% ethanol with centrifugation at 8000g. The tissue was lysed by incubation for a minimum of 12 h at 56°C with 160 μL of Buffer ATL (Qiagen) and 40 μL Proteinase K (Roche). The extraction was performed using semiautomated extraction on MagNA Pure LC platform (Roche). Extracts were stored at −80°C until testing.

Multiplex PCR was used for the detection of 19 different infectious agents in 8 different PCR reactions. Previously published PCR methods were used to detect *Ureaplasma urealyticum*, *Ureaplasma parvum*, *Mycomplasma genitalium*, and *Mycomplasma hominis* [18]. CMV, adenovirus, enterovirus, hepatitis C virus, herpes simplex virus types 1 and 2, human herpesvirus (HHV)-6, HHV-7, HHV-8, parvovirus B19, rubella virus, *Toxoplasma gondii*, and varicella zoster virus were detected using published methods [19].

*Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Actinobacillus actinomycetemcomitans* were detected by nested multiplex PCR using previously published primers targeting 16S rRNA [20, 21]. The Qiagen One-Step RT PCR kit (QIAGEN) was used for the first-round reaction mix in accordance with manufacturer’s instructions, and 200 nmol/L of each outer forward and reverse primer. Cycling conditions were 30 min at 50°C; 15 min at 95°C; 35 cycles of 45s at 94°C, 45s at 57°C, and 60s at 72°C; and 7 min at 72°C. The second round was performed using AmpliTaq Gold kit (Applied Biosystems) in accordance with the manufacturer’s instructions and 200 nmol/L of each inner primer. Second round cycling conditions were 5 min at 95°C; 35 cycles of 30s at 94°C, 45s at 57°C, and 45s at 72°C; and 10 min at 72°C.

Additional bacterial pathogens were identified by routine microbiological culture from autopsy. Positive PCR results were confirmed by repeated sectioning of paraffin blocks, nucleic acid extraction, and PCR, as described above. Plasmid constructs containing the outer (first-round) PCR products of each target gene were used as positive controls, whereas the negative controls contained water as template. Both controls were included in all PCR experiments.

**Immunohistochemistry**

Tissue sections of 4 μm were placed onto SuperFrost Ultra Plus silanized slides (Menzel-Glaeser) and immunostained using
murine monoclonal anti-CMV (Novocastra). This contains 2 monoclonal antibodies (clones DDG9 and CCH2) that bind to CMV immediate early protein (p72, clone DDG9) and the delayed early DNA binding protein (p52, clone CCH2) [22, 23]. All samples were processed using Bond Polymer Refine Detection system in an automated Bond immunostainer (Vision Biosystems). The primary antibody DDG9/CCH2 was applied at 1:100 dilution. A poly-horseradish peroxidase anti-mouse IgG (Novocastra) was used to localize the primary antibody and 3,3-diaminobenzidine was used as substrate chromogen to visualize the complex as brown precipitate, with hematoxylin-eosin counterstain. As a control, adjacent tissue sections were allowed to react with normal mouse serum (dilution, 1:1000) instead of the primary CMV antibody. Sections of a kidney from a known CMV-infected patient served as a positive control. Negative controls were tissue sections (kidney, liver, and placenta) shown to be CMV negative by PCR and immunohistochemistry.

Statistical Analyses
Cases that were determined to be CMV negative by PCR and immunohistochemistry but positive for another infectious agent were excluded from further statistical analyses. No other single pathogen was found in a sufficient number of cases to allow for statistical analysis. Because of the markedly different characteristics of the agents identified (ie, bacterial, viral, and protozoan) grouping these cases into “other infections” for statistical analysis would be inaccurate and misleading. Maternal and fetal characteristics are reported as median (interquartile range) or number (%). Birth weight percentiles were determined for gestational age. Clinical maternal and fetal characteristics were compared in CMV-positive versus CMV-negative cases using the χ² test or Mann-Whitney U test, as appropriate (Table 2). Pathological findings were compared using the χ² test or Mann-Whitney U test, as appropriate (Table 4). Associations between histopathological findings and presence or absence of CMV infection are reported in the text as the odds ratio and 95% confidence interval. Analyses were performed in SPSS, version 15 (SPSS Inc), and statistical significance was defined as P < .05.

RESULTS
A total of 130 singleton sequential stillbirth cases were selected and examined for the presence of CMV and other pathogens in this study. CMV DNA was detected in 20 (15%) of all 130 stillborn fetuses examined, whereas other infectious organisms were detected in 21 (16%) (Table 1). The next most frequently detected pathogens were Escherichia coli (4 of 130) (Table 1) and F. nucleatum and group B Streptococcus species (3 cases each) (Table 1). Although infection with organisms other than CMV was reasonable frequent (16%), no other single pathogen was present in >4 cases (3% of cases). Therefore, these cases involving other infections were excluded from further analyses.

<table>
<thead>
<tr>
<th>Target infectious agent</th>
<th>Proportion of organism-positive cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>1/130 (0.8)</td>
</tr>
<tr>
<td>Human cytomegalovirus</td>
<td>20/130 (15)</td>
</tr>
<tr>
<td>Enterococcus speciesb</td>
<td>1/130 (0.8)</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>1/130 (0.8)</td>
</tr>
<tr>
<td>Escherichia coli b</td>
<td>4/130 (3)</td>
</tr>
<tr>
<td>Fusobacterium nucleatum</td>
<td>3/130 (2)</td>
</tr>
<tr>
<td>Group B Streptococcus speciesb</td>
<td>3/130 (2)</td>
</tr>
<tr>
<td>Group A Streptococcus speciesb</td>
<td>1/130 (0.8)</td>
</tr>
<tr>
<td>Human herpesvirus-7</td>
<td>1/130 (0.8)</td>
</tr>
<tr>
<td>Human herpesvirus-8</td>
<td>1/130 (0.8)</td>
</tr>
<tr>
<td>Mycoplasma genitalium</td>
<td>1/130 (0.8)</td>
</tr>
<tr>
<td>Mycoplasma hominis</td>
<td>2/130 (1.5)</td>
</tr>
<tr>
<td>Toxoplasma gondii</td>
<td>1/130 (0.8)</td>
</tr>
<tr>
<td>Total</td>
<td>40/130 (31)</td>
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</tbody>
</table>

NOTE. a Target organisms screened for but not detected in any cases (0/130): Actinobacillus actinomycetemcomitans, Epstein-Barr virus, hepatitis C virus, herpes simplex virus (types 1 and 2), parvovirus B19, Porphyromonas gingivalis, rubella virus, Ureaplasma urealyticum, Ureaplasma parvum, and varicella zoster virus.

Approximately one-half of the mothers (52%) with a CMV-positive or CMV-uninfected stillborn infant were primiparous, and there were more male (59%) than female (41%) stillbirths. Table 2 shows demographic characteristics stratified by the presence or absence of CMV, as determined by PCR, immunohistochemistry, and microbiological culture.

Immunohistochemistry was performed by immunostaining (with CMV antibody) all of the CMV PCR-positive tissue specimens (n = 26) from the 20 CMV-infected stillbirths, along with CMV PCR-negative liver (n = 33), kidney (n = 33), and placenta (n = 33) specimens. Detection of CMV by immunohistochemistry was less sensitive than PCR (Table 3). Representative immunohistochemistry results of CMV-positive kidney, liver, and placenta specimens are shown in Figure 1, whereas common histopathological abnormalities are shown in Figure 2. CMV staining consistently showed cells containing CMV antigens with nuclear and cytoplasmic distribution. Some cells were cytologically modified after death, causing distortion of the normal architecture of the kidney and liver, resulting in the lack of distinctive cellular features in these organs. In the placenta, infection was most commonly localized in the chorionic villi. One PCR-negative liver tissue section tested CMV positive by use of immunohistochemistry, possibly because focal infection and sampling resulted in an insufficient CMV DNA template for successful PCR.

CMV was detected by PCR and immunohistochemistry in all the tissues examined (fetal kidney, liver, and placenta) for 2 stillborn infants. The autopsy report for the first case identified signs of infection in the amniotic fluid with a mild to moderate...
maternal inflammatory response and early-stage fetal inflammatory response. There was meconium staining in the amniotic fluid and extensive lymphohistiocytic villitis in the placenta. The placenta had extensive vascular sclerosed villi with residual T lymphocytes. The infant had extramedullary hematopoiesis, petechial hemorrhages, congenital pneumonia, hydrops, and congestion of the leptomeningeal vessels, and the possibility that the cause of death was due to viral infection was noted in the report. The second stillborn infant with disseminated CMV infection (based on PCR and immunohistochemistry results) had histological evidence of fetal thrombotic vasculopathy and intrauterine meconium exposure. There was evidence of stromal hemorrhage of villi, villous vascular proliferation, and petechial hemorrhages. Although intervillous space was reduced, there was no significant maternal underperfusion with respect to the gestational age. The postmortem report did not specify any evidence of infection, such as inflammation.

There were no statistically significant differences between the birth weight, birth weight centile, gestational age, or maternal parity in CMV-infected versus CMV-uninfected cases of stillbirth, although there were some missing data: parity was unknown for 2 of 20 CMV-infected and 16 of 89 CMV-uninfected stillbirths (Table 2). Notably, birth weight percentile for gestational age was 50th percentile in 75% of all stillbirths in this study group. Although it is not unexpected that stillborn infants would, on average, have a lower birth weight than live-born infants at the same gestational age, it also indicates that reduced birth weight is not exclusively associated with CMV infection. There was some evidence for an association between CMV infection and either early (<26 weeks) or term (>37 weeks) stillbirths \((P = .06; \text{data not shown})\); however, this finding did not reach statistical significance. There is also evidence suggesting a female sex bias toward CMV infection and stillbirth \((P = .06)\) (Table 2).

The pathological findings from postmortem examinations of stillborn infants from the CMV-infected and CMV-uninfected groups were compared (Table 4). Fetal thrombotic vasculopathy \((P = .010)\) was clearly specific to the presence of CMV in fetal tissue or placenta (Table 4). CMV infection was significantly more likely in cases of stillbirths with fetal thrombotic vasculopathy (odds ratio, 3.6; 95% confidence interval, 1.3–9.9). No other postmortem histopathological findings were associated with CMV infection in stillborn infants.

Table 2. Clinical Maternal and Fetal Characteristics of the Stillbirth Study Dataset

<table>
<thead>
<tr>
<th></th>
<th>CMV-positive cases ((n = 20)^a)</th>
<th>Uninfected cases ((n = 89)^b)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal parity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9 (45)</td>
<td>48 (54)</td>
<td>(P = .42)</td>
</tr>
<tr>
<td>2</td>
<td>4 (20)</td>
<td>13 (15)</td>
<td></td>
</tr>
<tr>
<td>&gt;3</td>
<td>5 (25)</td>
<td>12 (13)</td>
<td></td>
</tr>
<tr>
<td><strong>Fetal characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8 (40)</td>
<td>56 (63)</td>
<td>(P = .06)</td>
</tr>
<tr>
<td>Female</td>
<td>12 (60)</td>
<td>33 (37)</td>
<td></td>
</tr>
<tr>
<td>Gestation, median weeks (IQR)</td>
<td>34 (26–37)</td>
<td>31 (27–37)</td>
<td>(P = .84)</td>
</tr>
<tr>
<td>Birth weight, median g (IQR)</td>
<td>1860 (693.5–2670)</td>
<td>1445 (700–2425)</td>
<td>(P = .56)</td>
</tr>
<tr>
<td>Birth weight centile, median (IQR)</td>
<td>24 (5–49)</td>
<td>18 (2–49)</td>
<td>(P = .65)</td>
</tr>
</tbody>
</table>

**NOTE.** CMV, cytomegalovirus; IQR, interquartile range; PCR, polymerase chain reaction.

\(a\) CMV infection detected using nested PCR of all tissues, as described in the text.

\(b\) Determined by in the fetal tissues and placenta by PCR or microbiological culture during autopsy.

Table 3. Detection of Cytomegalovirus (CMV) DNA and Protein in Fetal Tissue (Kidney and Liver) or Placenta From Stillborn Infants

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>Proportion of CMV-positive results (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>By nested PCR</td>
</tr>
<tr>
<td>Kidney only</td>
<td>5/130 (4)</td>
</tr>
<tr>
<td>Liver only</td>
<td>6/130 (5)</td>
</tr>
<tr>
<td>Placenta only</td>
<td>5/130 (4)</td>
</tr>
<tr>
<td>Kidney and liver</td>
<td>2/130 (2)</td>
</tr>
<tr>
<td>Kidney, liver, and placenta</td>
<td>2/130 (2)</td>
</tr>
<tr>
<td>Total</td>
<td>20/130 (15)</td>
</tr>
</tbody>
</table>

**NOTE.** IHC, immunohistochemistry.

\(a\) One case had positive IHC results for the kidney sample and negative IHC results for the liver sample.

\(b\) One case had positive IHC results for the kidney and placenta samples and negative IHC results for the liver sample.

DISCUSSION

The etiology of up to one-half of all stillborn infants born in Australia and most developed countries is unknown [4]. CMV
infection is relatively common, has a high rate of transplacental transmission [13], and has been associated with placental damage that is known to result in fetal malformation and intrauterine death [25].

In this study, CMV DNA was detected in 15% of stillbirths examined (Tables 1 and 3). This incidence corresponds with recent reports that identified CMV in 16% of placentae from intrauterine deaths [26], although of the 15% CMV-positive stillbirths in the study, only 5% of placentae were CMV positive (Table 3). The incidence of CMV in fetal tissue and placenta is slightly higher than in other reports [15, 27], including a previous study from our group in which CMV DNA was detected in

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**Figure 1.** CMV protein in fetal organs and placentae from stillborn infants. Formalin-fixed, paraffin-embedded sections of kidney (top), liver (middle), and placenta (bottom) were analyzed using monoclonal mouse anti-CMV p72 and p52 (clones CCG9 and CCH2) by immunohistochemistry. After localizing the primary antibody with horseradish peroxidase anti-mouse immunoglobulin G, sections were incubated with diaminobenzidine (brown staining) and counterstained with hematoxylin-eosin (H&E), as described in the text. Representative fetal sections were brown in the presence of anti-CMV antibody (left), no staining in the absence of primary anti-CMV antibody (middle) and H&E (right). (Original magnification, ×400.)
4.3% of placentae from 2 unselected prospective cohorts (women undergoing elective amniocentesis and women attending antenatal clinics) [28]. The study inclusion criteria can play a significant role in detection rates, with the present study selectively investigating stillborn infants. In addition, we used solid-organ tissue samples and not amniotic fluid (from which CMV may be eliminated in some cases or virus detected after a delay in transmission from the maternal circulation) [27] or only placenta [26]. Furthermore, liver and kidney are 2 of the most frequently CMV-infected organs, in addition to the lung and pancreas [29]. Because of the nature of this study group (retrospective, with access to fetal autopsy specimens only), no data regarding maternal serostatus was available. In future studies, maternal serostatus would be of interest to evaluate any association with primary CMV infection versus CMV reactivation and stillbirth.

CMV antibody testing of placenta and other tissues was not performed as part of the routine postmortem examination in this study, because CMV infection was not suspected at the time of autopsy. We used immunohistochemistry to assess CMV viral protein expression and localize CMV infection in the infected chorionic villi of the placenta, including the villous cytotrophoblasts and syncytiotrophoblasts. In the fetal kidneys and liver, CMV had nuclear and cytoplasmic distribution. We observed more stillbirths that were CMV positive by PCR than by immunohistochemistry (Table 3), possibly as a result of the high sensitivity of nested PCR [30]. This discrepancy between PCR and immunohistochemistry may also result from (1) focal CMV infection [31, 32], (2) timing of fetal infection during gestation resulting in variable CMV dissemination in the fetus, (3) different fetal immune responses affecting viral load in different tissues [33], or (4) tissue degeneration reducing the number of intact cells for CMV immunohistochemistry analysis. Histopathology alone may not be able to provide an accurate measure of infection or cause of death in some cases of stillbirths but does demonstrate tissue inflammation and changes.

The effects of CMV infection on histopathological changes observed during postmortem examination showed that CMV infection was significantly associated with fetal thrombotic vasculopathy (Table 4), which is the presence of thrombi in the fetal circulation resulting in the clustering of fibrotic villi, characterized by absence or degeneration of fetal capillaries in contiguous villi [34]. This is consistent with CMV infection of endothelial and vascular cells and may potentially result in placental damage or altered placental permeability with increased transplacental transmission. Recent studies of immunocompromised and immunocompetent adults with active CMV infection have shown that CMV is a significant risk factor for the development of thrombosis [35, 36]. Fetal thrombotic vasculopathy has been reported in ~15% of placenta from stillbirths [37], compared with 43% in this study (Table 4). However, this may be due to the high incidence of CMV (15%), because the relationship with congenital CMV infection has not been previously examined. The etiology of fetal thrombotic vasculopathy remains unclear; however, umbilical cord abnormalities have previously been associated [24]. Fetal thrombotic vasculopathy has also been observed in placentae after other viral infections, such as severe acute respiratory syndrome [38]. Although we found an association between CMV infection and fetal thrombotic vasculopathy in stillborn infants, this is not sufficient to construct a profile of histopathological characteristics of CMV-associated stillbirth. Therefore, molecular testing for CMV—and possibly other viral infections—is an important technique for accurate postmortem evaluation of stillborn infants.

Other autopsy findings, such as villitis, chorioamnionitis, and maternal ischemia, have been associated with stillbirth in general [39, 40]. They were also frequently identified in this study (Table 4), and there was a tendency toward an association between villitis and CMV infection. However, this did not reach statistical significance, most likely because of the small number of cases observed. Histiocytic intervillositis, which has previously been associated with stillbirth [41, 42], was a very rare event in this study, with only 1 case in each group (Table 4). The possible association with female sex observed in this study...
(Table 2) may correlate with a previous report that female fetuses with congenital CMV infection have significantly increased risk of abnormal brain development [43]. A significantly larger number of stillbirth cases may be required to identify any possible associations between CMV infection and these less prevalent conditions.

The variability in associations between stillbirth and histological characteristics may result from (1) differential cytokine activation [44]; (2) viral infection occurring significantly prior to fetal death, by which time histological features in the placenta may have resolved in some cases; (3) differential CMV receptor expression on different placental cells [45]; and (4) focal CMV infection in the placenta [31, 32]. However, analysis of a larger study group with respect to CMV, fetal sex, gestational age, and the associated histopathological findings would help to clarify these interesting observations.

Stillbirth has multiple etiologies and several potential pathogenic mechanisms that could result in the death of the fetus. We demonstrate in this study that CMV infection has a strong association with stillbirth of unknown etiology after autopsy. The increased occurrence of fetal thrombotic vasculopathy with CMV infection is a possible CMV-mediated mechanism of fetal damage. The high number of stillbirths with CMV infection in this study does not prove an etiological association between CMV and stillbirth, but our findings support the value of using molecular testing as part of routine investigation for CMV infection in stillborn infants, because there are minimal histopathological characteristics that can be uniquely associated with CMV infection. Additional studies to evaluate CMV in placental and fetal tissue from stillbirths with a known genetic cause of death would further confirm the association of CMV with stillbirth. Accurate measures of CMV infection in pregnancy and the subsequent role of CMV in stillbirth are essential to properly assess the risk of recurrence, develop prenatal diagnostic recommendations, and optimize counseling for future pregnancies.

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**Acknowledgments**

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**References**

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