MAJOR ARTICLE

Distribution of Varicella-Zoster Virus DNA and Gene Products in Tissues of a First-Trimester Varicella-Infected Fetus

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Precise information about varicella-zoster virus (VZV) infection in first-trimester fetuses remains sketchy. After varicella infection was diagnosed in a woman, her 12-week-old fetus was aborted and was investigated, by histological examination, virus culturing, polymerase chain reaction, in situ hybridization (ISH), and immunohistochemistry (IHC), for the presence of VZV infection. Only the results of the histological examination suggested the presence of α-herpesvirus infection, in the gastrointestinal tract and liver; results of ISH were positive for VZV, and results of IHC staining were positive for intermediate early protein 63 (IE63) but negative for glycoprotein E (gE), in the dorsal root ganglia (DRG), meninges, gastrointestinal tract, pancreas, smooth muscle, liver, and placental trophoblast, indicating the presence of a nonproductive, latency-like VZV infection. Only the gastrointestinal tract and liver exhibited simultaneous staining for IE63 and gE, a result suggesting that active replication of VZV was present. In conclusion, widespread nonproductive VZV infection in the absence of histological clues is an early event in VZV infection in fetuses. The observed gene-expression pattern in most tissues resembles that of latent VZV infection in DRG. Latency-like infection in nonneural cell types may potentially reactivate, leading to multifocal necrosis, fibrosis, and dystrophic calcifications, as observed in advanced congenital varicella syndrome.

Primary varicella-zoster virus (VZV) infection during the first 2 trimesters of pregnancy may result in congenital varicella syndrome (CVS); the highest risk is ~2%, during weeks 13–20 [1]. CVS may either be limited to a single organ, such as the eye [2] or the skin [3], or involve the skin, the neural system, the gastrointestinal tract, the urogenital tract, the eyes, the muscles, and other organs. Multiple lesions and malformations frequently lead to fetal death [4, 5]. VZV DNA, detected by polymerase chain reaction (PCR), as well as VZV antigens, detected by immunohistochemistry (IHC), have been identified in various organs, including the lung, liver, skin, brain, and adrenal glands [6–10]. The association between certain congenital anomalies and virological and serological evidence of prenatal VZV infection has proved that maternal varicella infection causes CVS. But information on the distribution of VZV DNA and gene products in the tissues of first-trimester fetuses early in the course of fetal infection is lacking. Therefore, we used a combination of histological examination, PCR, in situ hybridization (ISH), and IHC to study VZV infection in a 12-week-old fetus.

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PATIENT, MATERIALS, AND METHODS

The mother provided informed consent to publish information about her and her fetus.

Histology. Routine histological examination of samples of the placenta and fetal tissues was performed on formalin-fixed, paraffin-embedded hematoxylin-eosin–stained sections. Fetal tissue samples were examined for evidence of α-herpesvirus infection, such as multinucleated giant cells and intranuclear inclusions; the placenta was examined for evidence of VZV infection, such as multifocal chronic villitis with multinucleated giant cells.

Table 1. Primer/probe system for detection of varicella-zoster virus (VZV) DNA.

	Nucleotide sequence, 5′→3′	Location, nucleotides
Primer		
VZV-gE(u)	ATACGGGCATCCACGTTATC	116193–116212
VZV-gE(d)	GCAAAAAGCTCCAAGTCTCG	116417–116398
Probe		
VZV-(A)	CTACGTTAAACGGCGATGACAGACATA	116214–116240
VZV-(D)	AATTGTAAATGTGAACCAACGTCAATACG	116242–116270

NOTE. (A), acceptor dye LC-red 640 coupled to the 5′ end of the VZV probe; (d), downstream primer; (D), donor dye fluorescein coupled to the 3′ end of the VZV probe; gE, glycoprotein E; (u), upstream primer.

Serology and virus culturing. Maternal serum was tested for VZV-specific IgM and IgG antibodies, by EIA (Enzygnost; Dade Behring) performed according to instructions supplied by the manufacturer of the kit that was used. RF-Absorbent (Dade Behring) was used to remove IgM rheumatoid factors from the serum before the sample was tested for the presence of IgM antibodies. In addition, an in-house IgG-avidity assay was performed; avidity was determined by a dissociation technique using the avidity reagent for the Enzygnost EIA. For virus culturing, a fetal tissue sample was inoculated into permissive human embryonal lung fibroblasts that were subcultured after 2 weeks. All cultures were observed for 4 weeks, to determine whether cytopathogenic effects were present.

Extraction of DNA for PCR. Before DNA was extracted from them, tissue samples were pretreated with proteinase K (0.5 mg/mL, in 10 mmol/L Tris-HCL [pH 7.6], 10 mmol/L NaCl, and 2 mmol/L EDTA) for 2 h at 37°C. Extraction of DNA from the liquefied tissue was performed on a MagNA Pure LC automated instrument, by use of a MagNA Pure LC Total Nucleic Acid isolation kit (Roche Diagnostics). The extracted DNA was thus highly purified and ready for use in PCR.

Amplification of VZV DNA by real-time PCR. VZV DNA was detected by use of a real-time PCR protocol and a LightCycler (LC) instrument. Amplification primers were chosen to amplify a 203-bp fragment of the gene for viral glycoprotein E (gE). The PCR master mix was based on a ready-to-use kit (LC FastStart DNA Master Hyb Probes; Roche Diagnostics) containing FastStart *Taq* polymerase and 2′-deoxynucleoside 5′-triphosphates mix. The mix was supplemented with $MgCl_2$ (4 mmol/L); uracil-N-glycosylase (UNG) (1 U); an upstream primer designated "VZV-gE(u)" and a downstream primer designated "VZV-gE(d)" (0.5 μmol/L each); and both a probe fluorescently labeled with acceptor dye LC-Red 640 (Roche Diagnostics) at the 5′ end of VZV and designated "VZV-(A)," and a probe fluorescently labeled with the donor dye fluorescein coupled to the 3′ end of VZV and designated "VZV-(D)" (0.1 and 0.2 μmol/L, respectively). After 15 μL of this mixture was pipetted into LC capillaries, 5 μL of the crude neucleic-acid preparation was added and the PCR run was started. For PCR, the following temperature profile was used: a 5-min step at room temperature, to enable UNG activity, and a 10-min step at 94°C, to inactivate UNG and to activate the FastStart *Taq* DNA Polymerase, before cycling. Cycling comprised 10 cycles of the following: denaturation (5 s at 95°C), annealing (15 s at 65°C and with a 1°C/cycle decrease from 65°C to 55°C), and extension (25 s at 72°C), followed by 45 cycles of denaturation (10 s at 95°C), annealing (20 s at 55°C), and extension (15 s at 72°C). Sequences of primers and probes are given in table 1.

Antibodies and nucleic-acid probes. The characteristics of (1) the polyclonal antibody (Pab) against IE63; (2) the monoclonal antibody (Mab) against IE63; (3) VL8, the antibody against gE; (4) antibody against 1U1 (Argene Biosoft); (5) the antibodies against IE63 that were used to detect various VZV-gene products; (6) the Pabs against herpes simplex virus (HSV)–1 and HSV-2; and (7) the DNA probes used in the ISH assays for VZV, HSV-1, and HSV-2 have been published elsewhere [11].

IHC. IHC was performed according to previously published protocols [11]. In short, primary-antibody incubation was performed by use of dilutions and incubation times that have been published elsewhere [11]. Subsequently, the slides were washed in Tris-buffered saline (TBS) and incubated for 30 min with the secondary antibodies (biotinylated swine anti-rabbit antibody diluted 1:300 and biotinylated rabbit anti-mouse antibody diluted 1:400; Dakopatts). Slides were rinsed in TBS and were covered by use of a polymer-based revelation system (EnVision; Dakopatts). After the slides had been rinsed in TBS, Fast Red (Dakopatts) was used as chromogen substrate for 5 min. The last steps consisted of counterstaining with Mayer's hemalun and mounting in Glycergel mounting medium (Dakopatts).

ISH. ISH was performed as described elsewhere for the *Eco*RI-(A) restriction-endonuclease fragment (in which acceptor dye LC-Red 640 is coupled to the 5′ end of *Eco*RI) [11]. The biotinylated probes for VZV, HSV-1, and HSV-2 [11] were used as follows: 5-μm sections were deparaffinized and then were treated, for 15 min at 37°C, with a solution containing proteinase K (10 μg/mL; Boehringer-Mannheim). The sections then were incubated, for 30 min at 37°C, in 10 μL of the hybridization

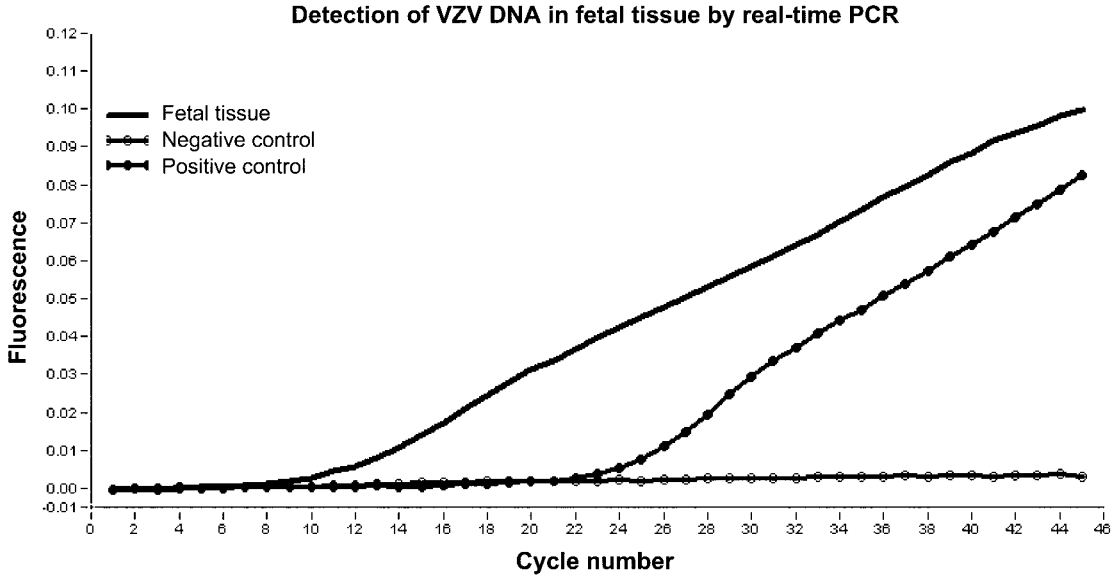

Figure 1. Detection of varicella-zoster virus (VZV) DNA in fetal tissue. Primers and probes were designed to enable fluorescence-resonance-energy transfer. The accumulating fluorescence *(vertical axis),* which is obtained by amplification of the 203-bp fragment of the gene for viral glycoprotein E, is acquired once per cycle *(horizontal axis)* and is displayed as a separate graphed line for each sample type. Although the positive control (●), containing an intermediate number of copies of VZV DNA, crosses the baseline fluorescence value at cycle 24, the negative control (○) remains at baseline values throughout the entire polymerase chain reaction. The remaining graphed line (i.e., that which does not include symbols), which is obtained by amplification of DNA extracted from fetal tissue, intersects the baseline fluorescence values, which already are apparent at cycles 10-12, a result indicating the presence of a high viral burden.

mixture containing 50% deionized formamid, 10% dextran sulfate, 15% 20 × SSC (3 mol/L chloride, 0.3 mol/L citrate), and 25% distilled water. Subsequently, the probe mixtures were incubated (1 μg/mL each of biotinylated VZV, HSV-1, and HSV-2 nucleic-acid fragments, in 50% deionized formamid, 10% dextran sulfate, and 15% 20 × SSC). After denaturation for 3 min at 90°C, the hybridization reaction continued for 60 min at 37°C. Slides were rinsed in TBS and were washed in a buffer (2 × SSC) for 30 min at 37°C. Alkalin phosphatase–coupled avidin biotin was applied for 30 min, and nitroblue tetrazolium and bromochloroindolylphosphate (Dakopatts) then were applied for 60 min. Sections were counterstained with Fast Red and were mounted in Glycergel.

Controls. Control specimens consisted of normal healthy skin and fetal tissue resulting from abortion. Positive control specimens included vesicular HSV and VZV skin infections from previous studies [11]; in IHC, ISH, and PCR, either the omission of primary-antibody incubation, secondary-antibody incubation alone, and/or probe/primer incubation served as a negative control.

RESULTS

Case history and laboratory findings. A 31-year-old, healthy, gravida 1, para 0 woman started her last menstrual period on 27 February 2003. On 8 May 2003, she presented with the classic clinical symptoms of chickenpox. In a Stuttgart laboratory (Labor Enders, Institut für Virologie, Infektiologie und Epidemiologie, Stuttgart, Germany), pimary maternal VZV infection was serologically confirmed, by both an elevated IgM titer (IgM index, 8.3; cutoff index, 1.0) and a low IgG-avidity index (10.8%). At both 10 weeks 4 days gestation and 11 weeks 1 day gestation, fetal ultrasound showed no abnormalities; but fetal death was diagnosed at 12 weeks gestation, and the woman was admitted to a hospital in Hagen (Department of Obstetrics and Gynecology, Allgemeines Krankenhaus, Hagen, Germany), so that labor, induced by prostaglandin, and subsequent curettage could be performed. Fetal/placental tissue specimens were analyzed by conventional histopathological examination (Department of Pathology, Allgemeines Krankenhaus, Hagen, Germany). The initial routine histological examination of these specimens revealed no signs that would suggest that α-herpesvirus infection was present. At the same time, some of the tissue specimens were send to the Stuttgart laboratory, for virus culturing and VZV PCR; no VZV was isolated, but the results of real-time PCR of fetal products were found, on the basis of a semiquantitative estimation, to be highly positive for VZV DNA (figure 1). To rule out the possibility that contamination by VZV DNA from maternal tissues or fluids could have occurred during curettage, formalin-fixed sections of tissue samples from the placenta and from the fetus were send to a Liège

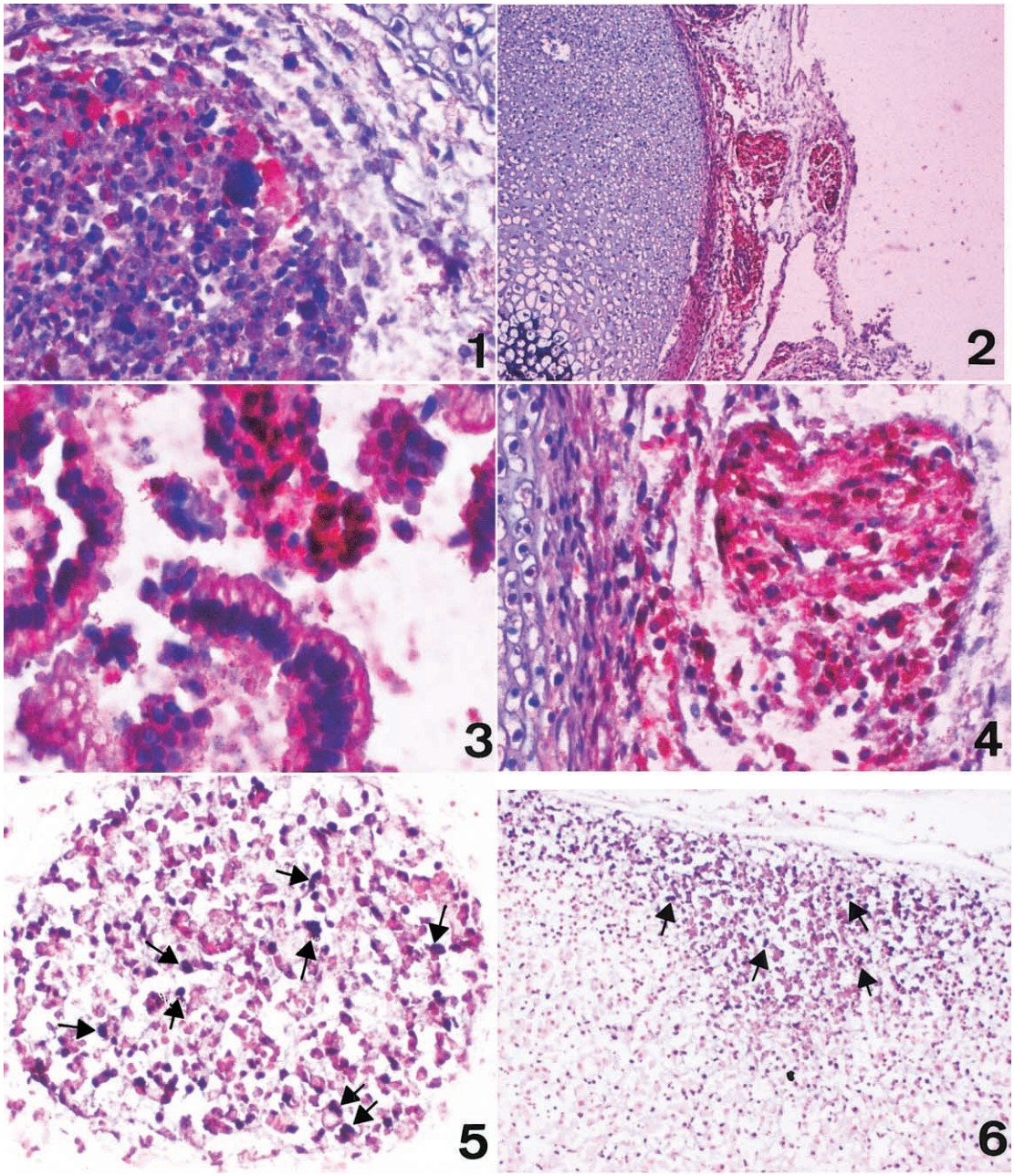

Figure 2. *1,* Positive results of immunostaining for varicella-zoster virus (VZV) intermediate early protein 63 (IE63) in fetal dorsal root ganglia (*red signal* [×40]). *2,* Positive results of immunostaining for VZV IE63 in a fetal dorsal root ganglion (*red signal* [×100]). *3,* Positive results of immunohistochemistry (IHC) staining for VZV IE63 in a fetal dorsal root ganglion (*red signal* [×100]). *4,* Positive results of IHC staining for VZV glycoprotein E in the digestive tract (*red signal* [×100]). *5,* Positive results of in situ hybridization (ISH) staining for VZV in fetal liver (*dark-blue nuclear signal [indicated by arrows]* [×40]). *6,* Positive results of ISH staining for VZV in a fetal dorsal root ganglion (*dark-blue nuclear signal [indicated by arrows]* [×100]).

laboratory (Department of Dermatopathology, University Hospital Sart Tilman, Liège, Belgium), for further investigation (by use of ISH and IHC). When the histological slides were reviewed in the context of the positive VZV-PCR results, cytolytic areas were observed both in tissue specimens from the liver and in those from the gastrointestinal tract. Both the IE63 Pab and the IE63 Mab showed a strong nuclear and cytoplasmic IHC signal in the cells of various tissue samples (figure 2). The antibodies against late (L) gE and L VZV showed prominent membranous and weak cytoplasmic immunostaining. ISH gave

Table 2. Varicella-zoster virus (VZV): comparison of histological results, immunohistochemistry (IHC) results, and in situ hybridization (ISH) results.

Tissue	Histological results[a]	IHC results, expression of IE and L antibodies			ISH results, signals	
		Abs to gE and to 1U1	Mab and Pab to IE63	Pabs to HSV-1 and to HSV-2	*Eco*RI-(A) and VZV	HSV
Fetus						
Striated muscle	−	−	+	−	+	−
Smooth muscle	−	−	+	−	+	−
Pancreas	−	−	+	−	+	−
Adrenal cortex	−	−	+	−	+	−
Spinal cord	−	−	+	−	+	−
Dorsal root ganglia	−	−	+	−	+	−
Gastrointestinal tract	+	+	+	−	+	−
Liver	+	+	+	−	+	−
Placental trophoblast	−	−	+	−	+	−
Villosities	−	−	+	−	+	−
Control						
VZV	+	+	+	−	+	−
HSV	+	−	−	+	−	+
Normal healthy skin	−	−	−	−	−	−

NOTE. (A), acceptor dye LC-Red 640 coupled to the 5′ end of *Eco*RI; Abs, antibodies; gE, glycoprotein E; HSV, herpes simplex virus; IE, intermediate early; L, late; Mab, monoclonal antibody; Pab, polyclonal antibody; +, positive; −, negative.

[a] Evidence of α-herpesvirus infection.

a positive nuclear signal in infected cells of the fetal and placental tissue samples. The histological, ISH, IHC, and control data are summarized in table 2. For several major organs (fetal brain, heart, lung, and spleen), tissue specimens could not be retrieved from the available paraffin-embedded sections.

DISCUSSION

To our knowledge this is the first report that describes the distribution of VZV DNA and gene products in placental and fetal tissues at an early stage of fetal VZV infection. The placental trophoblast and the villosities did not exhibit the microscopic alterations typical of viral infection; however, ISH showed evidence of VZV DNA in the placenta, and, in addition, IE63 was identified by IHC. These results call for complementary viral identification in placental tissue samples when clinical evidence of viral infection is present, even when histological evidence of α-herpesviruse is absent. It is indeed possible that a fetus could be aborted before such histological alterations can be observed. A productive VZV infection is biologically characterized by both (1) expression of the IE gene and the L gene and (2) histologically recognizable cytopathic effects [11]. Latent-VZV–infection patterns in human DRG are linked to the sole expression of IE-gene products—such as IE63, IE62, and IE4—and of some E-gene products, as has been demonstrated in rats [12] and humans [13]. In the present case, tissue specimens from some organs (e.g., the gastrointestinal tract and the liver) show cytopathic effects and are associated with positive results of ISH, as well as IE- and L-gene product expression, which suggests that active replication of VZV is present. Other tissues exhibit VZV DNA associated with gene expression restricted to some IE genes (table 2). This expression pattern resembles the latent-gene–expression pattern in DRG, or it could be viewed as a kind of abortive infection that has been described by Lungu et al. [14]. Our findings demonstrate that histopathological examination may underestimate the extent of viral dissemination in a fetus. Furthermore, primary VZV infection during pregnancy may result in fetal infection more often than previously has been thought. That the number of organs showing productive VZV infection (i.e., positive results of immunostaining for IE63 and for gE) is far less than the number of organs showing nonproductive VZV infection (i.e., positive results of immunostaining for IE63 but negative results of immunostaining for gE) possibly explains the limited sensitivity of VZV culturing [15].

On the basis of the clinical pattern of CVS, it has been suggested that the pathogenic mechanisms depend on the initial endogenous VZV reactivation in sensory ganglia of the posterior roots of the spinal cord [16]. But several manifestations (e.g., multifocal necrosis in parenchymatous organs and multiorgan distribution of dystrophic microcalcifications) of complex CVS are suggestive of a nonsegmental distribution of VZV infection. In the fetus examined in the present study, numerous nonneural organs showed ISH signals positive for VZV that were associated with IHC results showing strong expression of IE63. This observation may support the concept of an early disseminated infection of susceptible nonneural cells and local

VZV reactivation with consecutive tissue damage, a concept that has been proposed by others [17]. Early disseminated VZV infection in the absence of an adequate immune response to VZV has also been demonstrated in human adults [18]. Some investigators have found evidence, based on the detection of VZV DNA, of VZV persistence in nonneural fetal tissues [6, 8, 10]. Furthermore, an ongoing (>20 weeks) active inflammation around viral inclusions has been demonstrated in a fetus with CVS [9]. It is not clear whether the presence of VZV DNA and the expression of IE protein without the expression of gE possibly lead to cellular damage. Chronic inflammation may be similar to postzoster granulomatous reactions in which only VZV gE is evidenced, suggesting a delayed hypersensitivity reaction to persistent glycoproteins [19]. However, involvement of this mechanism in varicella infection in the first-trimester fetus in the present study is unlikely, both because IE63 but not gE was predominantly expressed and because the fetal immune system was immature. Whether a latent gene-expression pattern might result in specific pathological changes in infected cells remains to be elucidated.

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