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Identifying the etiologic role of Parvovirus B19 in non-immune hydrops fetalis by histopathology, immunohistochemistry and nucleic acid testing: a retrospective study

Koray Ergunay[1*], Gulcin Altinok[2], Bora Gurel[2], Ahmet Pinar[1], Arzu Sungur[2], Sevim Balci[3], Semsettin Ustacelebi[1]

[1] *Hacettepe University, Faculty of Medicine, Department of Clinical Microbiology, 06100 Ankara, TURKEY*
[2] *Hacettepe University, Faculty of Medicine, Department of Pathology, 06100 Ankara, TURKEY*
[3] *Hacettepe University, Faculty of Medicine, Department of Pediatrics, Division of Clinical Genetics, 06100 Ankara, TURKEY*

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Abstract: Intrauterine Parvovirus B19 infections may cause fetal anemia, non-immune hydrops fetalis or abortion. This study focuses on the pathogenic role of Parvovirus B19 in non-immune hydrops fetalis at Hacettepe University, a major reference hospital in Turkey. Twenty-two cases of non-immune hydrops fetalis were retrospectively selected out of a total of 431 hydrops fetalis specimens from the Department of Pathology archieves. Paraffine embedded tissue sections from placental and liver tissues from each case were evaluated by histopathology, immunohistochemistry, nested PCR and commercial quantitative Real-time PCR. Viral DNA was detected in placental tissues by Real-time PCR in 2 cases (2/22, 9.1%) where histopathology also revealed changes suggestive of Parvovirus B19 infection. No significant histopathologic changes were observed for the remaining sections. Nested PCR that targets the VP1 region of the viral genome and immunohistochemistry for viral capsid antigens were negative for all cases. As a result, Parvovirus B19 is identified as the etiologic agent for the development of non-immune hydrops fetalis for 9.1% of the cases in Hacettepe University, Turkey. Real-time PCR is observed to be an effective diagnostic tool for nucleic acid detection from paraffine embedded tissues. Part of this study was presented as a poster at XIIIth International Congress of Virology, San Francisco, USA (Abstract V-572).

Keywords: Hydrops fetalis, non-immune hydrops fetalis, Parvovirus B19, immunohistochemistry, nested PCR, Real-time PCR.

* E-mail: ekoray@hacettepe.edu.tr

1 Introduction

Vertical transmisson of Parvovirus B19 from a viremic mother to fetus may result in fetal anemia, non-immune hydrops fetalis, abortion or intrauterine death [1, 2]. When acquired early in pregnancy, the virus induces spontaneous abortion, and infection in the second trimester may result in non-immune hydrops fetalis. There is approximately a 30% risk for viral transmission to the fetus and an estimated $5-10\%$ overall risk of an abnormal outcome after a maternal infection. Parvovirus B19 accounts for $15-25\%$ of cases of non-immune hydrops fetalis; the etiology remains unknown in $20-50\%$ of all hydrops fetalis cases [3–5]. There is also enough evidence to suggest that the Parvovirus B19 infection is responsible for some of the intrauterine fetal losses in the late second and third trimesters [6]. Viral infections of cardiac myocytes have been documented, which may also contribute to cardiac malfunction [7]. Malformations resulting from intrauterine viral infection are not common features of Parvovirus B19, with only a few cases reported [8–11].

This retrospective study is designed to identify the etiologic role of Parvovirus B19 in the pathogenesis of non-immune hydrops fetalis cases observed at the Hacettepe University Ihsan Dogramaci Pediatric Hospital, a major pediatric hospital and reference center in Turkey.

2 Statistical methods and Experimental Procedures

2.1 Study Population

All pediatric autopsies performed after informed consent in the Hacettepe University School of Medicine Department of Pathology between January 1, 1980 and December 31, 2002 were retrospectively reviewed and 431 cases of hydrops fetalis were identified. After all other causes of hydrops fetalis - such as congenital heart anomalies, Rh-Rh isoimmunization, and genetic disorders - were ruled out by clinical and obstetric records and relevant laboratory data, 22 cases of non-immune hydrops fetalis were retrieved and included in the study. Sections from paraffine-embedded placenta and liver tissues from 22 non-immune hydrops fetalis cases were evaluated.

2.2 Detection of parvovirus B19 infection

The presence of Parvovirus B19 infection was investigated by histopathologic examination, immunohistochemistry, nested PCR and quantitative Real-time PCR for each section obtained from these selected non-immune hydrops fetalis cases.

2.3 Histopathology

1 to 4 different sections of hepatic and/or placental formaline-fixed paraffine-embedded tissues from each case were re-evaluated by histopathological examination after staining with hematoxylene and eosin by an experienced pathologist. Detection of nuclear inclusion bodies was considered to be suggestive of Parvovirus B19 infection [12].

2.4 Immunohistochemistry

All sections from the selected non-immune hydrops fetalis cases were evaluated for viral capsid proteins by mouse monoclonal antibodies against Parvovirus B19 VP1 and VP2 capsid proteins (Batch #: 109704, Novocastra Laboratories, UK) according the manufacturer's instructions as described before [13].

2.5 Nucleic acid extraction, nested PCR and real-time PCR

All tissues were deparaffinized and extracted by phenol-chloroform method using standard protocols as previously described [14, 15]. Three to four 5 μm thick sections from selected paraffine-embedded tissues were subjected to nucleic acid extraction in 1.5ml sterile tubes. Microtome blades were cleaned or changed after each sample processing. Excess paraffin was discarded with sterile scalpels if required. All purified nucleic acids were stored at -80^0C until amplification.

Ten microliters of the purified nucleic acids were used for nested PCR targeting the VP1 region of the viral genome in a 50 μl PCR reaction mix [16]. Oligonucleotide primers used in the first round of nested PCR were 5'-CTT TAG GTA TAG CCA ACT GG-3' (sense primer, nucleotides 2905-2931) and 5'-ACA CTG AGT TTA CTA GTG GG-3' (anti-sense primer, nucleotides $4016 - 3997$, yielding a 1112 base pair product. Second round PCR was performed using 5'-CAA AAG CAT GTG GAG TGA GG-3' (sense primer, nucleotides $3187 - 3206$) and 5'-CCT TAT AAT GGT GCT CTG GG-3' (anti-sense primer, nucleotides $3290 - 3271$) to produce a product of 104 base pairs (16). Preparations of PCR reaction mix and thermocycling conditions were performed as previously described [16]. All PCR reactions were performed on an MJ Research PTC-200 Peltier Thermal Cycler (MJ Research Inc., USA). PCR products were subjected to electrophoresis in 2% agarose gel and the expected amplicons were observed under ultraviolet light after staining with ethidium bromide.

Quantitative detection of viral DNA in purified nucleic acids was performed using the LightCycler Parvovirus B19 Quantification Kit (Roche Diagnostics, Germany) according to the manufacturers' instructions using a LightCycler 2.0 instrument (Roche Diagnostics, Germany) [17].

A positive serum from an acute Parvovirus B19 infection (viral load: 2000 copies/reaction) and commercial quantitation standards based on International Parvovirus B19 DNA standard were used as positive controls for nucleic acid-based tests. DNA extraction, prepara-

tion of PCR mixes and electrophoresis were performed in separate laboratories to prevent cross-contamination.

3 Results

A total of 28 placental and 23 liver sections from 22 non-immune hydrops fetalis cases were evaluated in the study. Clinical outcomes observed in the study group consisted of 14 therapeutic abortus and 8 intrauterine exitus. The male to female ratio was 12 to 10. Reported gestational age was between 22 to 40 weeks with a median of 32 weeks. The fetuses weighed between 815 to 2700 grams with a median weight of 1800 grams. No serologic data about maternal exposure to Parvovirus B19 was available.

3.1 Histopathologic Findings

Sections of placental tissues from cases No# 18 and 20 showed congestion, and capillaries within the villi were distended by nucleated red blood cells (Fig. 1). Intranuclear inclusions were detected within the nuclei of red blood cells (Fig. 2). Liver sections of these two cases displayed extramedullary hematopoiesis. No similar histopathologic changes were observed for the remaining cases (Table 1).

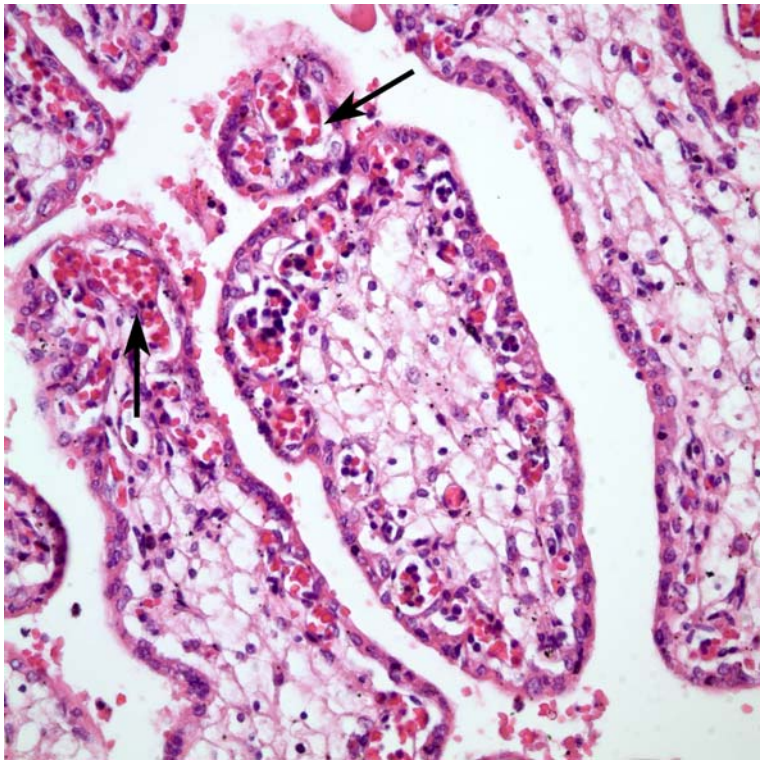

Fig. 1 Capillaries of the villi distended by fetal nucleated red blood cells (Placenta, HE stain).

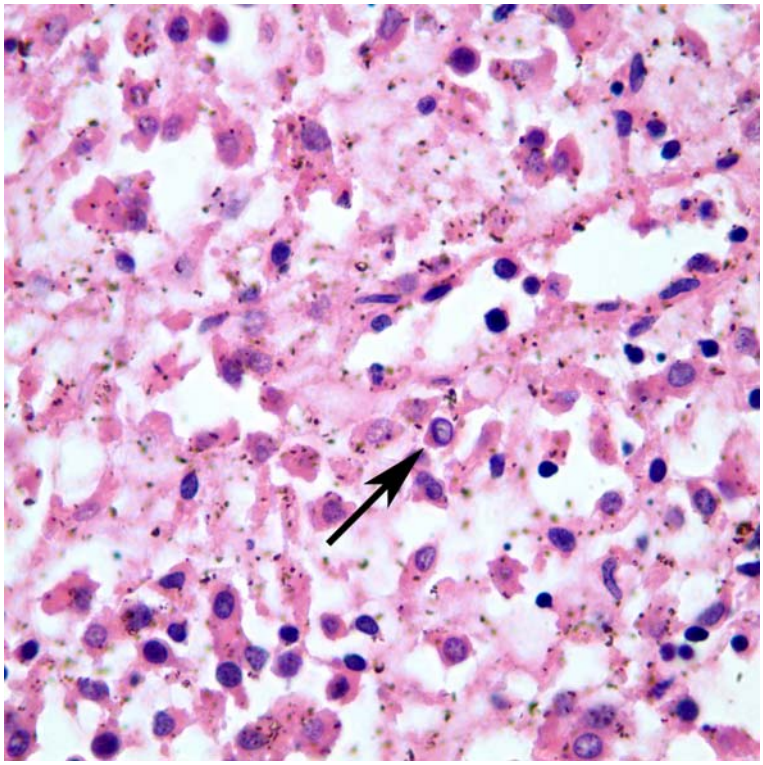

Fig. 2 Intranuclear viral inclusion in a nucleated red blood cell (Placenta, H&E stain).

3.2 Immunohistochemistry

All case samples were negative for viral capsid proteins by immunohistochemistry although the Parvovirus B19 positive control gave the expected staining pattern (Table 1).

3.3 Nested PCR

All samples were negative by VP1 nested PCR used in the study where expected amplicons were detected in positive control serum and Parvovirus B19 International Standard used as an alternate positive sample (Table 1).

3.4 Quantitative real-time PCR

Real-time PCR revealed positive results for 2 placental sections from cases No# 18 and 20, which also showed histopathologic changes suggesting intrauterine Parvovirus B19 infection. Both sections appeared to have low copy number of DNA ($<10^2$ copies/reaction; <20.13 IU/ml). All the remaining samples were negative (Table 1).

Table 1 Results of various techniques used for the detection of Parvovirus B19 induced fetal hydrops and distribution of sections evaluated in the study.

Cases	Sections		Results			
	#(Placenta)	#(Liver)	Histopathology	I.H.C[a]	Nested PCR	Real-time PCR
Case#1	1	1	not significant	negative	negative	negative
Case#2	1	1	not significant	negative	negative	negative
Case#3	1	1	not significant	negative	negative	negative
Case#4	1	1	not significant	negative	negative	negative
Case#5	1	-	not significant	negative	negative	negative
Case#6	2	2	not significant	negative	negative	negative
Case#7	2	2	not significant	negative	negative	negative
Case#8	1	1	not significant	negative	negative	negative
Case#9	1	-	not significant	negative	negative	negative
Case#10	2	1	not significant	negative	negative	negative
Case#11	1	3	not significant	negative	negative	negative
Case#12	1	1	not significant	negative	negative	negative
Case#13	1	1	not significant	negative	negative	negative
Case#14	1	1	not significant	negative	negative	negative
Case#15	1	1	not significant	negative	negative	negative
Case#16	1	1	not significant	negative	negative	negative
Case#17	1	1	not significant	negative	negative	negative
Case#18	3	1	not significant	negative	negative	negative
Case#19	1	1	not significant	negative	negative	negative
Case#20	2	2	S.C.[b]	negative	negative	Positive[c]
Case#21	1	1	not significant	negative	negative	negative
Case#22	1	1	S.C.[b]	negative	negative	Positive[c]

[a]: Immunohistochemistry
[b]: Suggested changes. Please refer to Results for description.
[c]: Positive for one placental section. Please refer to Results for details.

4 Discussion

Parvovirus B19 has been identified as one of the etiologic agents responsible for non-immune hydrops fetalis by various reports [1, 2, 6]. The aim of this study was to identify Parvovirus B19 as an agent responsible for development of non-immune hydrops fetalis at the Hacettepe University Ihsan Dogramaci Pediatric Hospital, one of the major reference centers in Turkey. It is known that the risk of fetal death due to Parvovirus B19 infection of mothers with confirmed evidence of primary infection is $5-10\%$ and the frequency of fetal loss due to Parvovirus B19 is estimated to be $4-16\%$ [2, 18–20]. No etiologic factor could be identified for 22 out of 431 (5.1%) fatal non-immune hydrops cases included in this study, and evidence of Parvovirus B19 infection was found in 2 (2/22, 9.1%). These two were Case 18 (23 weeks, male, 1900 grams, intrauterine death) and Case 20 (30 weeks, female, 1910 grams, intrauterine death). Both had histopathologic changes suggesting Parvovirus B19-related hydrops in placenta and liver tissues (Table 1). To our knowledge, this is the first report that addresses the role of Parvovirus B19 in hydrops fetalis by histopathology, immunohistochemistry and nucleic acid detection by nested and Real-time PCR from Turkey.

Parvovirus B19 is reported to be responsible for $15-25\%$ of cases of non-immune hydrops fetalis [3–5]. Evidence of Parvovirus B19 infection was observed in 9.1% in our

study (2 out of 22 cases). The underlying factors for this lower detection rate are not clear. An earlier exposure to Parvovirus B19, thus a lower prevalence of maternal infections, cannot be ruled out since there are no published reports on seroprevalence of Parvovirus B19 infections in Turkey. A thorough epidemiological survey is needed to clarify the prevalence of Parvovirus B19 infections in pregnant women in Turkey.

In this study, a standard immunohistochemistry method with commercially acquired monoclonal antibodies against viral capsid antigens, a commonly-used in-house nested PCR that targets viral capsid regions, and a commercial quantitative Real-time PCR system reported to have a good diagnostic performance were used to detect Parvovirus B19 infection [13, 17]. The use of paraffine-embedded tissues for molecular studies may be hindered by the effects of fixatives on target nucleic acids. Formaline overfixation may result in nucleic acid degradation and thus false negativity in molecular assays [14]. Since our study group was composed of cases that had already developed full-blown hydrops where viral replication was reported to be persistent, high viral loads and typical histopathological changes were expected to be observed for Parvovirus-induced hydrops [5, 21]. Despite these observations, we state that underestimation of the detection rate of Parvovirus B19 infection due to use of overfixed archival tissue was unlikely for the study. Our concordant results from histopathologic examination and Real-time PCR reveal that virally induced pathology can be detected with various techniques once hydrops had occurred. For positive cases, low viral loads, positivity of a limited number of sections from the same case might have been due to possible overfixation. Meanwhile, negative immunohistochemistry and in-house PCR results despite histopathological and Real-time PCR findings probably indicate negative effects of formaline overfixation. To overcome this problem, modified techniques of antigen retrieval for immunohistochemistry studies have been proposed [22]. For clinical decision-making and detection of the early phases of Parvovirus B19 infection, nucleic acid extraction from fresh tissue samples and amniotic fluid have been proven to be more effective [23]. Our results also indicate that Real-time PCR is an effective diagnostic tool, especially for qualitative nucleic acid testing from formaline fixed tissues, due to enhanced sensitivity and detection of smaller nucleic acid targets.

As a result of this retrospective analysis, Parvovirus B19 infection have been shown to be responsible for 9.1% of cases of hydrops fetalis in our group by histopathology and Real-time PCR.

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