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A Molecular Approach to Detect Type B Coxsackie Virus in Iraqi Patients with Different Types of Abortion

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ABSTRACT

Background & Objective: The infection in newborns can present in different ways, ranging from no noticeable symptoms to more serious conditions like meningoencephalitis, myocarditis, and disseminated sepsis. The present study was conducted with aim to evaluate the potential correlation between Coxsackie B Virus (CBV) infection and pregnancy loss within a cohort of Iraqi women.

Materials & Methods: cross-sectional study was conducted from November 2022 to June 2023. The study population included 100 pregnant women aged 15-40 years with gestational age ranging from 0 to 16 weeks. Each participant completed a standardized questionnaire. Afterward, placental tissue samples were collected from women who underwent elective termination of pregnancy. These samples were then analyzed for the presence of Coxsackievirus B (CVB) genome using a molecular biology approach. The detection process involved RNA extraction, cDNA, and qRT-PCR.

Results: Among 100 women included in this study, 88 had missed abortions, 10 had incomplete abortions, and 2 had spontaneous abortions. The mean age of the women was as follows: 28±2.8 years for missed abortions, 29±1.5 years for incomplete abortions, and 32±0.5 years for spontaneous abortions. The mean gestational age was 10±1.1 weeks for missed abortions, 10±1.5 weeks for incomplete abortions, and 14 weeks for spontaneous abortions. There was no presence of Coxsackie B virus nucleic acid in the placental tissues obtained from the women who had abortions. The internal control (quality control) and positive control both yielded positive results.

Conclusion: Despite the assay's high sensitivity for accurate detection, the study produced only null findings. These negative results are likely to be true negative, indicating the absence of the target analyte.

Keywords: Coxsackie B Virus (CBV), Missed abortion, Incomplete abortion, Parturient, Placental tissue

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1. Introduction

Maternal viral infections can cause serious birth defects or developmental issues in the developing embryos or fetus. For example, rubella, Cytomegalovirus (CMV), and Herpes Simplex Virus (HSV) are well-known teratogens that can lead to significant morbidity and mortality in newborns with congenital malformations. Other viruses such as parvovirus B19, enterovirus group, hepatitis B virus, and human immunodeficiency virus are also recognized as significant causes of adverse pregnancy outcomes. Coxsackievirus B (CVB) is a type of positive-strand RNA virus belonging to the Enterovirus genus in the Picornaviridae family. CVB consists of six different serotypes, known as CVB1 through CVB6 **(1)**.

Coxsackievirus intrauterine infection is primarily documented through indirect evidence that suggests transmission across the placenta. Neonates can present with a range of symptoms from no symptoms to severe conditions such as meningoencephalitis, myocarditis, and sepsis. The report by (Konstantinidou, et al) presents first documented case of prenatal findings and fetoplacental pathology in a third-trimester fetus with confirmed coxsackie B3 transplacental infection, using molecular techniques **(1,2)**.

In a recent investigation by Satosar et al., placental tissues of neonates with perinatal demise, severe morbidity, or both were evaluated for the presence of Coxsackievirus. Viral detection was achieved in 22 (36.7%) of the cases. Conversely, none of the control

placentae exhibited evidence of any infectious agent (3).

The role of the coxsackie virus is significant in causing adverse effects in full-term pregnancies, such as neonatal mortality or illness at birth. Miscarriage can result from various factors, including maternal age, chromosomal abnormalities, hormonal imbalances, abnormal blood clotting, infectious agents, and environmental factors (4).

Coxsackieviruses, specifically Type B, are classified as pathogens with the potential to induce a spectrum of diseases such as myocarditis, aseptic meningitis, and pancreatitis. The following highlights from the provided sources offer valuable insights to inform the molecular detection of Type B Coxsackievirus in Iraqi patients with diverse abortion types (5, 6). Studies have suggested a relationship between Coxsackie B4 and the onset of Type 1 diabetes (7, 8). This association emphasizes the importance of detecting and comprehending Coxsackievirus infections in different health conditions. Patients infected with Coxsackievirus B display increased levels of Coxsackievirus-B IgG antibodies, indicating exposure to the virus. This antibody response can serve as a critical indicator of past or current infections. Coxsackievirus B has been observed to exit host cells in shed microvesicles, which may contribute to the spread of the virus. Understanding the mechanisms of viral spread can assist in detecting the presence of the virus in various tissues (9-11).

Coxsackieviruses interact with both the innate and adaptive immune systems, evading and exploiting host responses (12). This interaction is crucial in understanding how the virus persists and spreads within the body. Molecular markers like the "fluorescent timer" protein have been used to track virus infection and dissemination in real-time (13, 14). Such tools can be valuable for studying the behavior of Coxsackievirus in different groups of patients. To detect Type B Coxsackievirus in Iraqi patients with different types of abortion, a molecular approach can be used. This approach involves analyzing specific markers, such as Coxsackievirus-B IgG antibodies, studying viral spread mechanisms, and utilizing advanced molecular tools for real-time virus tracking. By taking this comprehensive approach, valuable insights can be gained into the presence and impact of Coxsackievirus infections in patients with different health conditions, including various types of abortion (9-11).

A significant proportion (over 30%) of conceptuses in euploid females undergo spontaneous pregnancy termination during the initial stages of gestation. While chromosomal aberrations are implicated in roughly half of these early pregnancy losses, the etiology of a substantial number of spontaneous abortions remains obscure (15-17). The present study was conducted with aim to elucidate the potential correlation between Coxsackie B Virus (CBV) infection and pregnancy loss within a cohort of Iraqi women.

2. Materials and Methods

This cross-sectional study was conducted from November 2022 to December 2023. The study population included 100 women who experienced miscarriage in Baghdad, specifically at al-Imamain Kazemain Medical City, al-Karkh Maternity Hospital, and Al-Alawia Maternity Hospital in Baghdad Governorate. The women aged 15 and 40 years with gestation age of 0-16 weeks at the time of miscarriage, which could be missed, incomplete, or spontaneous. The clinical and laboratory data of the patients were collected from the consultant gynecologist. Women who tested positive for TORCH, UTI, or abnormal chromosomal anomalies were excluded from the study.

Ethical Approval

The current study adhered to ethical guidelines and was conducted in accordance with the consent form provided by the Iraqi Ministry of Health. Ethical approval for this study was granted by the Institutional Review Board (IRB) at Al-Nahrain University (reference number: 204-14/11/2022). Prior to participation in the research, written informed consent was obtained from all participants.

Samples Collection and Processing

Placental tissue specimens were obtained from each patient. To maintain the integrity of RNA, the freshly collected tissue was promptly immersed in Trizol reagent and stored at -80°C for subsequent RNA extraction, cDNA synthesis, and PCR analysis.

Viral genome detection

RNA Extraction

Ribonucleic Acid (RNA) purification was conducted using the commercially prepared Up Plus RNA extraction Kit (Abbexa abx098093/UK). This kit includes a set of reagents designed for the isolation of total RNA from cells and tissues. The homogenate was treated with chloroform and subjected to centrifugation, resulting in the separation of the solution into an aqueous supernatant enriched with RNA, an interphase, and a lower organic extract which was colored. The RNA in the supernatant was then efficiently isolated and purified using a spin column containing a silica matrix. The Up Plus RNA extraction Kit is known for its powerful lysis capability, which ensures higher purity and yield of RNA. This column-based methodology streamlines the entire process of cell disruption and RNA isolation.

Assay the purity and concentration of the extracted RNA

After isolating viral Ribonucleic Acid (RNA) from tissue specimens, a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) was used to measure

the quantity and assess the quality of the extracted RNA. So that, 1-2 µL aliquots of the purified RNA were loaded into the instrument's cuvette. If the samples had a 260/280 nm absorbance ratio between 1.7 and 1.9, they were considered suitable for further analysis. Otherwise, the RNA extraction process was repeated.

Complementary DNA (cDNA)

The RNA is converted into Complementary DNA (cDNA) through reverse transcription, using a commercially available reverse transcriptase enzyme. The GoScript™ Reverse Transcription System is a convenient kit that allows efficient synthesis of first-strand cDNA, which is then optimized for PCR amplification. The GoScript™ in vitro reverse transcription kit reagents can be used to reverse transcribe RNA substrates, including whole transcriptome RNA, polyadenylated messenger RNA, or in vitro transcribed RNA. Up to 5 µg of total RNA, poly(A)+ mRNA, or synthetic transcript RNA can be used as templates in 20 µL reactions with components from the GoScript™ Reverse Transcription System. The resulting first-strand cDNA samples are then ready for PCR gene amplification.

PCR amplification

Complementary DNA (cDNA) samples were amplified using the Quantitative Polymerase Chain Reaction (qPCR) kits provided by Bioingentech® (Chile). These kits consist of the One Universal qPCR DNA Master Mix, which is a pre-optimized reaction mixture containing all the necessary reagents for efficient qPCR. The kit includes specific primers for the Coxsackievirus B that can be detected using the TaqMan probe-based detection method on your qPCR instrument. Coxsackie B virus detection through PCR involves targeted hybridization, where specific forward and reverse primers bind to complementary sequences within the target genomic DNA of Coxsackie B. The reaction mixture also contains a fluorogenic probe, a single-stranded DNA molecule labeled with two fluorophores: a reporter molecule (FAM) at the 5' end and a quencher molecule at the 3' end. The reporter emits fluorescence in the green spectrum, which can be measured to monitor the amplification process.

To validate the extraction of a legitimate nucleic acid target, endogenous reference amplification reagents are incorporated. These reagents comprise an oligonucleotide probe labeled with a fluorophore reporter molecule (HEX) and a quencher molecule, which hybridize within a specific reference gene. The PCR reaction mixture consists of an isolated DNA template, oligonucleotide primers, fluorescently labeled probes, and internal amplification control. Nuclease-free water and a master mix containing reaction buffer, dNTPs, and a DNA polymerase were added in volumes of 3.4 µL, 0.6 µL, 3.4 µL, and 7.5 µL, respectively. This mixture was prepared identically for both positive and negative controls. The PCR was then conducted following a predetermined thermal cycling protocol (Table 1). The analysis of the results was performed according to the manufacturer's instructions (Table 2).

Table 1. Thermo cycling profile of PCR

Cycle	Steps	Time	Temp (°C)
1 cycle	Activation step	1 min	95°C
40 cycles	Denaturation	30 sec	95°C
	Annealing	30 sec	58°C
	Extension	30 seg	72°C
	Final step	∞	4°C

Table 2. Ct value result

Ct value	Results
0-11	Negative
12-36	Positive
36-40	Marginal Positive
> 40	Negative

3. Results

A total of 100 aborted women were recruited (88 missed abortion, and 10 incomplete abortion, 3 spontaneous abortion). Mean maternal ages were 28±2.8, 29±1.5 and 32±0.5 years in missed abortion, incomplete abortion, and spontaneous abortion respectively. Mean gestational ages were 10±1.1, 10±1.5, and 14 weeks in missed abortion, incomplete abortion, and spontaneous abortion, respectively (Table 3).

Coxsackie B virus nucleic acid was not found in placental tissues obtained from women who had undergone different types of abortions. The average Cycle Threshold (Ct) values for the internal control (quality control) were 28±2.8, 29±1.5, and 32±0.5 in missed abortion, incomplete abortion, and spontaneous abortion, respectively (Table 4). The absence of target amplification in the sample, Non-Template Control (NTC), and negative control (Table 5) confirms that the negative results are valid. On the other hand, the positive amplification observed in internal control and positive control demonstrates that the assay is functioning properly.

Table 3. Characteristics of enrolled aborted women

	Missed abortion (n88)	incomplete abortion(n10)	Spontaneous abortion(n2)
Mean maternal age (yrs)	28±2.8	29±1.5	32±0.5
Mean gestational age (weeks)	10±1.1	10±1.5	14

Table 4. Prevalence of coxsackie b virus in placentas tissues by q-Real time PCR

	Missed abortion	Incomplete abortion	Spontaneous abortion
Mean of internal control (quality control) CT	23±1.6	24±1.9	23±1.9
Mean of sample CT	- (0/88) No CT	- (0/10) No CT	- (0/2) No CT

Table 5. Interpretation of results

Sample	NTC	Internal control (quality control)	Positive control	Negative control	The results
Negative (no CT)	Negative (no CT)	Positive	Positive	Negative (no CT)	True negative

4. Discussion

Coxsackieviruses A and B are uniquely found in humans and transmitted among individuals through the fecal-oral route and possibly via respiratory aerosols. The pediatric population is particularly vulnerable to coxsackievirus infection, leading to a wide range of clinical manifestations affecting various organ systems. These systems encompass the integumentary (skin), mucosal surfaces, cardiovascular system, gastrointestinal tract, central nervous system, and peripheral nervous system **(1, 18)**. Although in utero transmission of Coxsackievirus B (CV-B) represents a significant public health concern yet remains under-recognized and inadequately characterized. Vertical perinatal transmission is the most common mode, but both in utero and post-partum dissemination via maternal and non-maternal routes have been described in the literature **(19)**.

The results of our study indicate that Coxsackie-B virus was not found in the placental tissues of any of the women who had abortion. Additionally, the rate of missed abortion was higher compared to other types of abortion, while spontaneous abortion had the lowest percentage among all types studied. It is worthnoting that the mean maternal age in the spontaneous abortion group was the highest (32±0.5 years).

There are multiple hypotheses that can be proposed to elucidate these results. Our findings provide evidence suggesting a potential decrease in viral prevalence within this particular patient cohort. Alternatively, the data may imply an increased vulnerability to the effects of the virus during the third trimester of gestation. These observations align with previous research conducted in this area. Several studies have reported that vertical transmission of coxsackievirus during late pregnancy or parturition can lead to severe neonatal enteroviral infection **(19, 20)**.

This study presents the first comprehensive description of fetal pathological findings associated with confirmed intrauterine transplacental transmission of Coxsackievirus B3. Maternal infection manifested during the third trimester with a significant decrease in fetal activity **(21)**.

Furthermore, there is limited case studies documenting Congenital Viral Botulism (CVB) infection within the human fetus during the first trimester of pregnancy **(22)**. However, another study showed that infection during the second week of pregnancy had negative effects on both the mother (dams) and the outcome of the pregnancy (increased rates of stillbirth and abortion, as well as reduced litter size). Additionally, the timing of the infection during pregnancy affected the severity of postnatal infection in the offspring when they were exposed to the same pathogen.

Alternatively, it is plausible that there may be localized variations or focal alterations within the placental structures. The placenta fulfills a pivotal role in protecting the fetus, but it may also be susceptible to damage due to its function as the primary avenue of exchange. Our observations are supported by in vivo studies conducted on laboratory animals. Notably, the brain exhibited high concentrations of Coxsackievirus B3 (CVB3), indicating CVB3 infection has a substantial impact on the development of cardiac malformations **(23)**.

However, the findings of our study disagree with the results of certain prior investigations concerning viral identification in the placenta and the umbilical cord. We have observed similar levels of the virus in these tissues as compared to those in fetal tissues. In fact, previous research has designated the placenta as a potential site for CV-B3 and CV-B4 **(24)**.

The sample size of the current study might be too small to detect the presence of the virus, which is why it was not observed. Increasing the sample population could potentially lead to positive cases and enhance the study's ability to identify the virus. Another possibility is that there is a stronger connection between viral dissemination and spontaneous miscarriage. However, because the miscarriage group had a limited sample size, the present study did not find any evidence to support this hypothesis.

The current hypothesis is in concordance with prior research **(25)**, who identified Coxsackievirus B2 (CVB2) in spontaneously aborted fetal tissue. Furthermore, another study provided evidence suggesting a causal link between CVB infection and spontaneous abortion **(26)**.

To detect type B Coxsackievirus in a cohort patient with different types of abortion, a molecular method for enterovirus typing can be utilized. This method allows for the identification of specific enterovirus serotypes like Coxsackievirus B. Coxsackievirus B5, for example, has been commonly reported among enterovirus and is associated with various clinical presentations, including hand, foot, and mouth disease. Additionally, Coxsackievirus B2 and B1 have been linked to clinical illnesses like aseptic meningitis, myocarditis, and neonatal systemic illness. These viruses have endemic patterns of circulation with specific activity periods **(27, 28)**. Moreover, studies have shown that enteroviruses, including Coxsackieviruses, may play a role in the pathogenesis of type 1 diabetes. Coxsackievirus B1 has been associated with an increased risk of β-cell autoimmunity, particularly when infection occurs shortly before the appearance of autoantibodies. On the other hand, Coxsackievirus B3 and B6 have been associated with a reduced risk β-cell autoimmunity, indicating potential immunological cross-protection against Coxsackievirus B1. These findings highlight the importance of considering the specific serotypes of Coxsackievirus in understanding their impact on health outcomes **(28)**. A molecular approach for enterovirus typing can help identify the specific type B Coxsackievirus in patients with different types of abortion. It is crucial to understand the characteristics and clinical associations of various Coxsackievirus serotypes, such as B1, B2, and B5 for accurate detection and management in clinical settings **(27, 28)**.

5. Conclusion

In conclusion, despite having high assay sensitivity and accuracy, the present study only produced null findings. These negative results are likely to be true negative. However, one possible limitation could have contributed to small sample size. It is recommended that our findings require a larger sample size and recruitment from a geographically diverse population. Additionally, future investigations should include testing for the virus in paternal tissues and a wider range of aborted samples, including fetal tissues and placental tissues. Furthermore, it is important to have a

balanced distribution of samples across all types of abortions to ensure the generalizability of the results.

Acronyms

CV-B: Coxsackieviruses serotype B; q-RT-PCR: qualitative real time polymerase chain reaction; Ct: cycle threshold

6. Declarations

Acknowledgments

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Ethical Considerations

There are no etical issues relevant to this work.

Authors' Contributions

Al-Obaidi and Abdulrasul designed the research study. Bassim M Jasim and Bassim H Jasim executed all experimental procedures, guidance on data collection techniques. Baqer A Al-Obaidi and Bassim M Jasim analysed the results and directed the research endeavor and curated the final written. Bassim M Jasim authored the initial manuscript. All authors reviewed and approved the final version of the manuscript for publication.

Conflict of Interest

There is no conflict of interest.

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