

## Infectious Agents and Miscarriage in Bulgaria

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### Abstract

A number of infection agents have been linked to miscarriage and to other adverse outcomes of pregnancy, such as stillbirth and preterm delivery. The purpose of present study was to determine the implication and prevalence of viral agents (parvovirus B19, rubella, CMV and adenoviruses) and *Chlamydia trachomatis* in the etiology of miscarriage during the first and second trimester of pregnancy. A total of 62 serum samples from women with miscarriage (n=32) and control group of healthy women (n=30) for period January 2015 – June 2016 were tested by ELISA (detected of specific IgM/IgG antibodies) and PCR (detected of specific genomic region) assays. The possible role of B19V, *Ch. trachomatis* and adenoviruses for miscarriages were detected in 6/32 (18.75%) by ELISA and in 7/32 (21,87%) by PCR methods.

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The seroprevalence of protective IgG antibodies in the highest level was proven against rubella 25/32 (78,12%) and the lowest – against adenoviruses (1/32, 6,25%). All tested healthy women in the control group had a negative result for acute infection for the five tested infectious agents. The detailed study aimed at enriching the diagnostic palette of these infectious pathogens is necessary for understanding the exact mechanisms behind infection-induced miscarriages and could lead to effective treatment and thus prevention.

**Keywords:** miscarriage; parvovirus B19; *Chlamydia trachomati*; ELISA; PCR.

## 1. Introduction

Abortions may be classified as early or late, spontaneous or induced for therapeutic or elective reasons, threatened or inevitable, incomplete or complete, recurrent (also called recurrent pregnancy loss), missed, or septic. Spontaneous abortion (miscarriage or pregnancy loss) is non-induced embryonic or fetal death before 20 week gestation and it could be early (before 12 gestation weeks) or late (from 12 to 24 gestation weeks) [1; 2]. The clinical diagnosis is based mainly clinical criteria (no fetal activity and loss of fetal heart activity) and pregnancy not located on ultrasound and treatment is usually observation or uterine evacuation [3; 2]. The incidence of miscarriage abortion is about 10 to 15% in confirmed pregnancies as a half of women with confirmed pregnancies bleed during the first 20 week of gestation have spontaneous abort [2].

Isolated spontaneous abortions may result from certain viruses (most notably cytomegalovirus, herpesvirus, parvovirus B19, and rubella virus) and bacterial agents caused a condition known as bacterial vaginosis (*Gardnerella vaginalis*, group B streptococci, *Staphylococcus aureus*, *Ureaplasma urealyticum* or *Mycoplasma hominis*, *Chlamydia trachomatis* and ets.) or from disorders that can cause sporadic abortions or recurrent pregnancy loss (eg, chromosomal or mendelian abnormalities, luteal phase defects) [4; 5; 6]. According to [2] pathogens and their association with miscarriage could be divided into three groups: associated little or no evidence for association and conflicting evidence for association with miscarriage. Other causes include immunologic abnormalities, major trauma, and uterine abnormalities (eg, fibroids, adhesions). The data from a number of studies [7; 8] indicate that 15% of early miscarriages and 66% of late miscarriages can be due to infections. The mechanisms of action of infectious agents which may result in spontaneous abortion may be divided into several groups: direct infection of the uterus, fetus, or placenta, placental insufficiency, or infected intrauterine device [9]. Using an enzyme-linked immunosorbent assay (ELISA) to detect specific antibodies in sera, as well as a standard amplification method (polymerase chain reaction, PCR) for detection of infectious nucleic acid in sera or tissues, a positive infectious association with miscarriage was observed [2].

The risk factors for miscarriage are: age > 35; cigarette smoking; use of certain drugs (eg, cocaine, alcohol, high doses of caffeine); a poorly controlled chronic disorder (eg, diabetes, hypertension, overt thyroid disorders) in the mother [10; 11; 12; 13; 14; 15] and also a previous miscarriage [16].

Therefore, the monitoring of pregnant women at risk (screening for IgG seroprevalence) and pathological pregnancy (detection of acute viral or bacterial infection) is a very important part of a successful outcome of pregnancy.

The purpose of present study was to determine the implication and prevalence of infectious agents in the etiology of miscarriage during the first and second trimester of pregnancy.

## **2. Materials and Methods**

### **2.1. Patients and specimens**

**Clinical samples.** A total of 32 serum samples from women with spontaneous abortion for period January 2015 – June 2016 were tested to detect possible infectious cause of fetal loss. In the study had included a control group of 30 healthy women screened for detection of immunity before their in vitro fertilization. The average age of patients studied was  $31,33 \pm 4,97$  years.

### **2.2. Serological analysis**

Blood samples were taken on admission by venipuncture from each patient and tested for anti-parvovirus B19 IgM/IgG, anti-Rubella IgM/IgG, anti-Adenovirus IgM/IgG, anti-Cytomegalovirus IgM/IgG and anti-Chlamydia IgM/IgG antibodies. Blood was centrifuged at 4000 g for 10 min and serum was aliquoted and frozen at  $-20^{\circ}\text{C}$  until analyzed.

All serum samples were tested for the presence of IgM/IgG antibodies with a commercial indirect enzyme-linked immunosorbent assay (Euroimmun, ELISA IgM/IgG kits). The assays were performed as recommended by the manufacturer and the results were interpreted qualitatively as positive, negative or equivocal.

### **2.3. DNA extraction and polymerase chain reaction (PCR)**

Viral DNA extraction was attempted from all serum samples using the PureLink®Viral RNA/DNA test kits.

The screening for B19V DNA was performed with primers e1905f and e1987r (20 p/mol) located in the NS1 gene (NS1-PCR) [17] and AmpliTaq Gold PCR Master Mix Kits. DNA of *Chlamydia trachomatis* were detected [18] also used AmpliTaq Gold PCR Master Mix Kits.

A standard One Step Reverse Transcription PCR (RT-PCR) procedure for the detection of rubella virus which was based on the amplification of the E1 region on rubella viruses, by means of two overlapping fragments and two pairs consensus primers were used [19]: Fragment 1 (480 bp): RV 8633F and RV 9112R and Fragment 2 (633 bp): RV 8945F and RV 9577R.

Detection of adenoviruses was carried out by the Real Time RT-PCR method with the use of a kit – SuperScript III Platinum ® One-Step Quantitative RT-PCR System (Invitrogen) and standard primers and probes. Amplification was performed with a Chromo 4 thermal cycler (Bio-Rad) according to the protocol of [20; 21]. A Ct value  $<38$  was regarded as positive.

**Table 1:** Number of samples tested by years and infectious agents and number of positives by tested serological IgM/IgG markers

Years	Tested infectious agents									
	No of Rubella virus ELISA IgM positive (%)	No of Rubella virus ELISA IgG positive (%)	No of B19V ELISA IgM positive (%)	No of B19V ELISA IgG positive (%)	No of CMV ELISA IgM positive (%)	No of CMV ELISA IgG positive (%)	No of <i>Ch. trachomatis</i> ELISA IgM positive (%)	No of <i>Ch. trachomatis</i> ELISA IgG positive (%)	No of AdenoV ELISA IgM positive (%)	No of AdenoV ELISA IgG positive (%)
<b>2015 (n=25)</b>	0 (0%)	19 (76%)	3 (12%)	12 (48%)	0 (0%)	23 (92%)	1 (4%)	5 (20%)	1 (4%)	2 (8%)
<b>to June 2016 (n=7)</b>	0 (0%)	6 (85,71%)	0 (0%)	2 (28,57%)	0 (0%)	7 (100%)	1 (14,28%)	2 (28,57%)	0 (0%)	0 (0%)
<b>Total (n=32)</b>	0 (0%)	25 (78,12%)	3 (9,37%)	14 (43,75%)	0 (0%)	30 (93,75%)	2 (6,25%)	7 (21,87%)	1 (3,12%)	2 (6,25%)
<b>Control group (n=30)</b>	0 (0%)	27 (90%)	0 (0%)	17 (56,67%)	0 (0%)	27 (90%)	0 (0%)	2 (6,67%)	0 (0%)	1 (3,33%)

## 2.4. Electrophoresis assays

The PCR products were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide.

## 3. Results

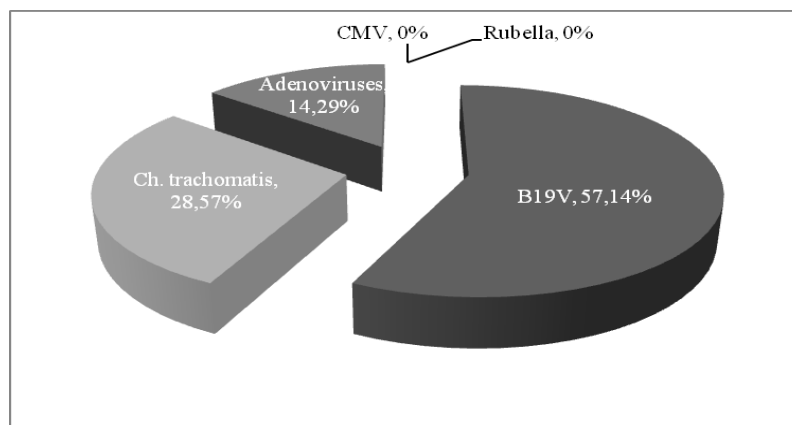
During the January 2015 – June 2016 a total 32 serum samples from women with miscarriage by indirect ELISA assays were tested. All samples were tested for the presence of specific antibodies (IgM/IgG) for five infectious agents with a potential role for fetal loss (Table 1).

The marker IgM antibodies indicating acute infection was observed in 6/32 (18,75%) for all period and by years 5/32 (15,62%) in 2015 and 1/32 (3,12%) in 2016. Positive ELISA IgM results were found in 3/32 (9,37%) for B19V, in 2/32 (6,25%) for *Ch. trachomatis*, and in 1/32 (3,12%) for adenoviruses, respectively. A recent rubella and CMV infections in the tested by ELISA women with adverse pregnancy outcomes were not found.

The presence of protective IgG antibodies is an important laboratory marker on one side in connection with proven meeting with an infectious agent and specific response of immune system to it, on the other hand the existence of immunity and protection of the body for a period of time or for life. Of all tested the highest percentage specific rubella IgG antibodies were found 25/32 (78,12%) and the lowest - adenoviruses IgG (1/32, 6,25%) (see Table 1).

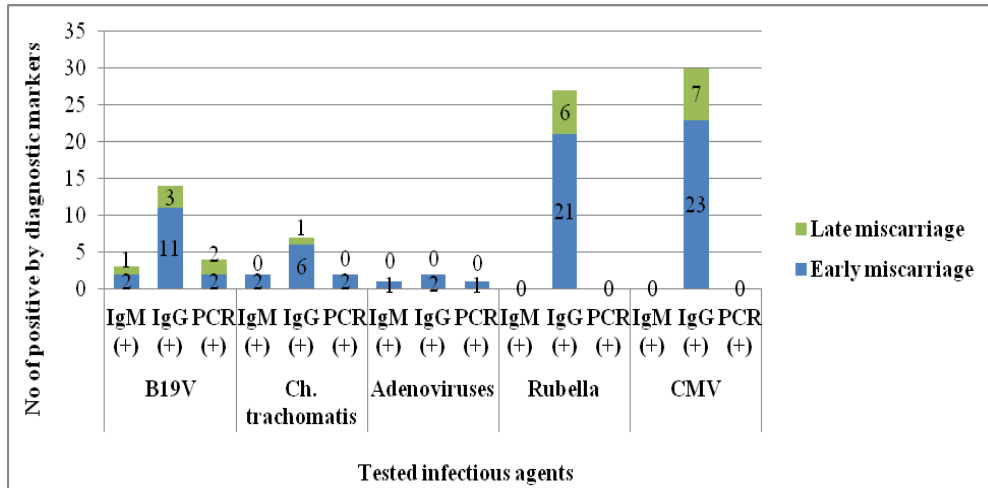
All tested healthy women in the control group had a negative result for acute infection for the five infectious agents. The seroprevalence of protective IgG antibodies in the highest level was proven against rubella, cytomegalovirus, and parvovirus B19, respectively.

All samples were examined for the presence of infectious nucleic acid by using a standard and real time PCR. Positive PCR signals were obtained in 7/32 (21,87%). In 4/7 (57,14%) PCR positive samples were detected B19V-NS1 region, in three of them had combination with positive B19V IgM/IgG results. *Ch. trachomatis* DNA were found in 2/7 (28,57%) which were *Ch. trachomatis* ELISA IgM positive specimens. Adenovirus ELISA IgM positive sample also had positive real time PCR signal (Figure 1).



**Figure 1:** Number of positive by molecular diagnostic assays in percentage

According to gestational age of fetal loss patients were divided into two groups: early miscarriage (before 12 weeks of gestation) and late miscarriage (12 - 24 weeks of gestation). The most tested and affected were in group of early miscarriage and in first trimester of pregnancy. By ELISA in 5/6 (83,33%) and by PCR in 5/7 (71,43%) were confirmed cases like early miscarriage. The other positive cases (1/6, 16,66% by ELISA and 2/7, 28,57% by PCR) were in the group of late miscarriage and were established positive for parvovirus B19 (Figure 2).



**Figure 2:** Number of positive by detected of a specific diagnostic markers (ELISA IgM/IgG antibodies and DNA/RNA specific regions) and a period of miscarriage

**4. Discussion**

The miscarriage is considered the most common adverse pregnancy outcome (approximately 15% of all clinically recognized pregnancies). An intrauterine infection plays a major role in the pathogenesis of early pregnancy loss to 20 weeks of gestation but the pathway and the mechanism of infection has not yet been well established.

In this study, we analyzed women with miscarriage during the first and second trimester (32 serum samples) and healthy women (30 serum samples) which had visited the Gynecological Hospitals in Bulgaria. The average age of patients studied was 31,33 ± 4,97 years. Specimens were investigated by serological and molecular methods for the presence of rubella, B19V, CMV, adenoviruses and *Ch. trachomatis* - pathogens associated with miscarriage [2].

The data in England and Wales for 2012-2013 shown fetal loss of one in five pregnancies or more of 200 000 miscarriages for this period [22]. In an Australian prospective cohort including 14 247 women aged 18–23 years, the rate of miscarriage varied from 11.3 to 86.5 per 100 live births amongst different groups; overall, miscarriage occurred in 25% of the women in the study when the women were 31–36 years old [23].

In Bulgaria there has been a trend to reduce the number of abortions (1990 – 144,644; 2000 – 61,378) and they account for half of births during the year. According to data during 2012 the number of abortions is 29 992

compared with 69 678 births. Of all of abortions performed by 2012 - 2930 were on medical indicators (9,77%), 9 821 were miscarriage (32,75%) and the other 17 240 (57,48%) were on optional [24].

Our study includes combinatorial analysis for several infectious agents on first or single samples taken to two weeks after the moment of spontaneous abortion. None of these agents could be found in control group of healthy women, only protective antibodies for rubella, CMV and B19V were found in them. We detected a possible role of B19V, *Ch. trachomatis* and adenoviruses for miscarriages in 6/32 (18.75%) by ELISA and in 7/32 (21,87%) by PCR methods.

In study from Italy six out of eight cases (more of 90%) of fetal loss observed were 'attributed to B19V infection' and was noted that the risk of vertical transmission is higher if infection occurs by gestational week 20 [25]. The same results reported [26] in Japan. In our study the total four B19V (12,5%) affected were found and most of them were in first trimester. Interestingly, B19V IgG antibodies were higher in controls than cases (56.67 and 43,75%, respectively), similar to the study of [27]. *Ch. trachomatis* is pathogens which is a known risk factor for ectopic pregnancy and preterm birth [28]. The positive association with miscarriage was observed on [29]. They were detected IgG antibodies against *Ch. trachomatis* in higher levels in the miscarriage group (15,2%) than in the controls (7,3%). In the present study we found 2/32 (6,25%) positive *Ch. trachomatis* samples for acute and 7/32 (21,87%) for recent infection. *Ch. trachomatis* has been studied extensively and a lot of data are available for this infection from over three decades of research. Contradicting studies have been published, resulting in conflicting evidence regarding the role of *Ch. trachomatis* in miscarriage [30; 31]. Taking into account the most recent findings and the increase in screening programs worldwide, such as the screening offered to all pregnant women in the USA (CDC, 2014), public awareness of the possible risk of *Ch. trachomatis* infection to a future pregnancy might be advisable [2]. Adenoviruses are pathogens with little or no evidence for association with miscarriage [2]. However, we received positive serological and molecular result for recent adenovirus infection in a patient with spontaneous abortion and data for respiratory infections. The assays applied in the present study do not cover all potential infectious causative agents of miscarriage (Bacterial vaginosis - *Mycoplasma hominis* and *Ureaplasma urealyticum*; *Brucellosis*; *Syphilis*; *Coxiella burnetii*; *Mycoplasma genitalium* and other viruses - Dengue fever, HIV, Hepatitis C, Human papillomavirus, Herpes simplex virus 1 and 2 and etc.) and also included a relatively small number of tested patients (32 women with miscarriage and 30 healthy women). Despite the so mentioned limitations of the study, the tested panel of carefully selected and analyzed samples during a period of 18 months, results described here, provides an evidence for the leading role of B19V, adenoviruses and *Ch. trachomatis* infections in the pathogenesis of miscarriage at the present time in Bulgaria.

## 5. Conclusion

From the above, it is evident that a many viral and bacterial agents play a key role in miscarriage. The combined diagnostic approach and case-control study on women with fetal loss is appropriate to detected of possible etiological agent, in therapy and prognosis, respectively.

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