

Human parvovirus B19 and cytomegalovirus infections, and thyroid autoimmunity in women with recurrent first trimester abortion

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ABSTRACT

Antithyroid antibodies, parvovirus B19 (B19V) and cytomegalovirus (CMV) infections were studied to evaluate their role in the pathogenesis of recurrent first trimester abortion, and to elucidate their importance in routine prenatal investigations of women with unexplained recurrent abortion. Sixty women with unexplained recurrent first trimester abortion were the study group and thirty term healthy pregnant women, were selected as a control. Placental and fetal tissues were analyzed for B19V and CMV deoxyribonucleic acids using nested polymerase chain reaction (PCR) assay. Sera were analyzed for T3, T4 and TSH levels, the presence of antithyroid antibodies (thyroglobulin and thyroid peroxidase), CMV specific IgM antibodies and parvovirus B19-DNA by dot blot hybridization assay. Parvovirus B19-DNA was demonstrated in nearly one third of the aborted products (19/60 = 31.7%) and 6.7% of the placentas of the control group ($P < 0.001$). Sera samples of the two groups were negative for parvovirus B19 viral DNA by dot blot hybridization. Cytomegalovirus - DNA was demonstrated in 7/60 aborted tissues (11.7 %) and one placenta of the control (3.3 %) ($P > 0.05$). In contrast, only 5 aborting women (8.3 %) and a woman of the control group (3.3%) were CMV-IgM seropositive ($P > 0.05$). Antithyroglobulin antibody levels were significantly higher in aborting women (7/60) than control ($P < 0.05$), whereas there was no significant changes in antithyroid peroxidase antibody levels between the two groups. Nine aborting women (15%) were seropositive for one or both of the thyroid autoantibodies compared with two (6.7%) of the control group ($P > 0.05$). We did not find any significant relation between thyroid autoimmunity and viral infections (B19V & CMV) in women with unexplained recurrent first trimester miscarriage. In conclusion, parvovirus B19 infection was found to be an important pathogenic agent in the case of recurrent first trimester abortion. It is essential to investigate for B19V infection among pregnant women with recurrent abortion. This significant finding demonstrates the usefulness of a sensitive molecular tool, PCR assay of parvovirus B19 DNA to aid in the diagnosis. However, testing for cytomegalovirus infection and antithyroid antibodies are not essential as routine prenatal investigations and should be restricted to certain patients with unexplained recurrent pregnancy loss.

RIASSUNTO

Infezioni umane da Parvovirus B19 e da Citomegalovirus, e autoimmunità tiroidea in donne con aborto ricorrente al primo trimestre

Al fine di valutarne il ruolo nella patogenesi nell'aborto ricorrente al primo trimestre e di chiarirne l'importanza nella sorveglianza prenatale delle donne con aborto ricorrente inspiegato, sono stati studiati gli anticorpi anti-tiroidei e le infezioni da parvovirus B19 (B19V) e da citomegalovirus (CMV). Sono state incluse nel gruppo di studio 60 donne con aborto ricorrente al primo trimestre; come controllo sono state incluse 30 donne gravide a termine sane. Nei tessuti della placenta e del feto è stato determinato il DNA del B19V e del CMV mediante reazione polimerasica a catena (PCR) "nested". Nei sieri si sono misurate le concentrazioni di T3, T4 e TSH e si è ricercata la presenza di anticorpi anti-tiroidei (tireoglobulina e perossidasi tiroidea), e di anticorpi IgM anti-CMV e anti-B19V mediante ibridizzazione "dot blott". B19V-DNA è stato dimostrato in circa 1/3 dei prodotti d'aborto (19/60 = 31,7 %) e nel 6,7 % delle placente del gruppo di controllo ($P < 0,001$). I campioni di siero dei due gruppi erano negativi per B19V-DNA. CMV-DNA veniva dimostrato in 7/60 materiali abortivi (11,7%) ed in una placenta del gruppo di controllo ($P > 0,05$). In contrasto solo 5 donne del gruppo di studio (8,3 %) e una del gruppo di controllo (3,3 %) erano CMV-IgM siero positive ($P > 0,05$). Gli anticorpi anti-tireoglobulina erano significativamente più alti nel gruppo di studio (7/60) che nei controlli ($P < 0,05$), mentre non vi erano differenze significative nei valori degli anticorpi anti-perossidasi tiroidea nei due gruppi. Nove donne del gruppo di studio (15 %) erano sieropositive per uno o entrambi gli anticorpi anti-tiroidei in confronto a due (6,7 %) del gruppo di controllo ($P > 0,05$). Non si è riscontrata nessuna reazione significativa tra la autoimmunità tiroidea e le infezioni virali (B19V & CMV) in donne con interruzione della gravidanza ricorrente inspiegata al primo trimestre. In conclusione, l'infezione da B19V è risultata essere un importante agente patogeno nel caso di aborto ricorrente al primo trimestre. È importante ricercare la infezione da B19V nelle gravide con aborto ricorrente. Questi significativi risultati dimostrano la utilità di uno strumento molecolare sensibile, la ricerca mediante PCR del B19V-DNA, come ausilio per la diagnosi. D'altro canto le ricerche della infezione da CMV e degli anticorpi antitiroidei non sono essenziali come usuale indagine prenatale e dovrebbero essere ristretti a speciali pazienti con interruzioni ricorrenti inspiegabili della gravidanza.

INTRODUCTION

Recurrent pregnancy loss is an intriguing problem in obstetric practice. Over the last decade, many etiologic factors and several treatment regimens have been reported. However, there is no definite evidence for most causes of recurrent miscarriage and therapeutic interventions of any kind can be considered as merely empirical (1).

Evidence supporting the autoimmune concept is now extensive. Triggering circumstances such as infection, endocrine, genetic, environmental, and cultural mechanisms, or ingestion of drugs as well as aging process appear to participate in the development of autoimmune diseases (2). Abnormal autoantibody levels can result in clinical, subclinical, or preclinical autoimmune conditions. Muller et al (3) reported that the presence of a large variety of autoantibodies was associated with repeated pregnancy loss. This association was first made with reference to lupus anticoagulant and other antiphospholipid antibodies (4). Thereafter, Stagnaro-Green et al (5) was the first to describe the association between antithyroid antibodies and pregnancy loss.

On the other hand, many viral infections appear to be involved in the etiology of spontaneous abortion: cytomegalovirus, rubella, herpes simplex, mumps and Epstein Barr virus (6, 7).

Although human cytomegalovirus (CMV) infection is the most common viral infection transmitted via the placenta (8), Parvovirus B19 can also be transmitted from the infected mother to the fetus. Therefore, spontaneous abortion, fetal anemia, nonimmune hydrops and stillbirth could result (9).

The present study reports the results of our investigations in apparently healthy women with at least two or more unexplained first trimester pregnancy losses. These women were screened for certain factors that are not routinely investigated in clinical practice of handling a case of miscarriage, aiming at assessing its clinical importance and demonstrating whether it should be a routine prenatal investigations in recurrent first trimester pregnancy losses. These women were screened for: (a) Antithyroid autoantibodies in serum; (b) Human parvovirus B19 (B19V) infection: viral DNA- was detected in serum and aborted products; (c) Cytomegalovirus (CMV) infection: specific-IgM antibodies measurement in maternal serum and viral DNA in the placenta and products of conception.

Detection of these viral infections was selected because the infected patients are frequently asymptomatic or exhibited symptoms commonly associated with pregnancy, such as fatigue and joint pain. We tried to clarify whether there will be any association between viral infections (CMV and parvovirus B19) and the presence of thyroid autoimmunity to determine their roles in the pathogenesis of recurrent first trimester pregnancy loss.

Subjects and Methods

One hundred pregnant women were selected from the obstetrics and emergency departments of Al-Zahraa Uni-

versity Hospital in the period from December 2000 and December 2001. They were classified into two groups:

Group I: thirty term pregnant women (37 - 41 weeks gestations) attending the delivery Department with uncomplicated pregnancy and with no history of pregnancy loss (control group).

Group II: seventy pregnant women in the first trimester of pregnancy with history of two or more recurrent abortions and presented with spontaneous abortion, missed or inevitable abortion, diagnosed by history, examinations and confirmed by ultrasound scanning (study group).

Most of the participants of the study group were selected from a larger pool of repeated aborters of women with obvious other causes for pregnancy loss had been excluded. Patients with significant uterine anomalies, uterine myomas, parental chromosomal abnormalities that were detected previously from peripheral blood, patients with possible luteal phase defect and autoimmune abnormalities among phospholipid antibodies were excluded.

The patients age, parity, occupation, obstetric history, date of last menstrual period, and description of any symptoms (fever, rash, or arthralgias), or history of exposure to child with erythema infectiosum were recorded. All women were subjected to the following:

A-Blood was drawn on admission and separated serum was stored at - 70 °C for determination of:

- 1-TSH ,T3 and T4 levels ,
 - 2-Thyroid autoantibodies: thyroid peroxidase (TPO) and thyroglobulin (TG) ,
 - 3- CMV specific IgM ,
 - 4- Parvovirus B19 DNA by dot blot hybridization assay.
- B-The products of conceptions of the miscarried women and the placenta of the delivering women were collected and stored at -70°C for nested PCR assay of: 1-Parvovirus B19-DNA. 2. Cytomegalovirus CMV - DNA.

NB: We preferred to study B19V - DNA in the aborted materials by PCR assay as Peters and Nicolaidis (10) demonstrated that IgM antibody determination is less sensitive than PCR assay and hence less useful tool for diagnosing intrauterine B19V infection. We also studied B19V- DNA in the serum by hybridization technique.

Laboratory evaluation

*TSH, Total T3 and thyroxin (T4) were determined by using enzyme linked immunosorbent assay method (Diagnostic System Laboratories , Inc, Texas , USA) (11). The procedure of ELISA followed the basic principle of enzyme immunoassay where there was competition between an unlabeled antigen and an enzyme labeled antigen for a fixed number of antibody binding sites. The amount of enzyme labeled antigen bound to the antibody was inversely proportional to the concentration of the unlabeled analyte present. Unbound materials were removed by decanting and washing the wells. The absorbance measured was inversely proportional to the concentration of TSH ,T3 and T4 present in the serum. Sets of TSH, T3 & T4 standards were used to plot a standard curves of

absorbance versus TSH, T3 & T4 concentrations and the unknowns can be calculated. The normal range for TSH was (0.35 - 5.5) uIU/mL active T4 is defined as 4.5 - 11.3 ug / dL and for total T3 as 69 - 202 ng / dL

***Thyroid peroxidase antibodies (TPO)** were assayed using Quanta Lite TM TPO kit (Inova Diagnostics, INC, San Diego) (12). Purified human TPO antigen was bound to the wells of a polystyrene microwell plate under conditions that will preserve the antigen in its native state. Prediluted controls and diluted sera were added to bind to the immobilized antigen. Unbound sample was washed away and an enzyme labeled antihuman IgG antibody was added to each well. A second incubation allows the enzyme labeled to bind to any patient antibodies that had become attached to the microwell. After washing away any unbound enzyme conjugate, the remaining enzyme activity was measured by adding a chromogenic substrate and measuring of the color that developed. The assay can be evaluated by comparing the color that developed in the patient wells with the color in the control wells. Negative values range from 0-100 units, positive results are > 100 units.

***Thyroglobulin Antibodies by chemiluminescent EIA**

Serum thyroglobulin antibodies were measured by IMMULITE Automated Analyzer (Spencer et al 1996). This method is a solid phase, two step chemiluminescent enzyme immunometric assay for use with the Immulite for the quantitative measurement of antibodies against thyroglobulin. The solid phase, a polystyrene bead enclosed within an Immulite test unit, is coated with highly purified thyroglobulin. The prediluted patient sample and the buffer matrix were simultaneously introduced into the test unit, and incubated for 30 minutes at 37°C with intermittent agitation. During this time, thyroglobulin autoantibodies in the sample bind to the thyroglobulin coated bead. The serum / buffer mixture was then removed by a centrifuge wash. An alkaline phosphatase-labeled antihuman IgG antibody was introduced, and the test unit was incubated for another 30-min. cycle. The unbound enzyme conjugate was removed by a centrifugal wash, after which substrate was added, and the test unit was incubated for a further 10 min. The chemiluminescent substrate, a phosphate ester of adamantyl dioxetane, hydrolysed in the presence of alkaline phosphate to yield a stable intermediate. The continuous production of this intermediate resulted in the sustained emission of light, thus improving precision by providing a window for multiple readings. The bound enzyme conjugate and thus also the photon output, as measured by the Luminometer was proportional to the concentration of thyroglobulin autoantibodies in the sample. The detection limits of the assays defined as the concentration two standard deviations above the response at zero dose, is approximately 3 IU/ml.

N.B: Elevated levels of antibodies to thyroid peroxidase (TPO antibodies) occur more frequently than high anti-

thyroglobulin levels in autoimmune thyroid disease. However, anti-thyroglobulin determination do not seem to add to the diagnostic information provided by anti-TPO results.

***Cytomegalovirus IgM antibodies by chemiluminescent EIA**

Qualitative detection of IgM antibodies to cytomegalovirus (CMV) in human serum by Immulite Automated Analyzer. Immulite CMV IgM is a solid phase, two step chemiluminescent enzyme immunoassay (13). The solid phase, a polystyrene bead enclosed within an Immulite test unit, is coated with partially purified CMV antigen. The procedure of detection is the same as that of anti-thyroglobulin. A qualitative result was obtained by comparing the patient result to an established cut off.

***Dot blot hybridization assay of parvovirus B19 DNA in sera**

The assay was performed according to Yoto et al (14). In brief, 20 µL of each serum mixed with 180 µL of distilled water, were filtered with manifold apparatus (Biometra,) onto a treated nylon membrane (Genescreen, BRL, USA). After the samples were filtered, the nylon membrane was air dried and baked for 2 hours under a vacuum at 80°C. The specimens on the filters were then alkali denatured, neutralized, treated with proteinase K and washed in 2xSSC buffer (0.3 M NaCl plus 0.03 M sodium citrate; pH 7.0). After prehybridization at 65°C for 2 hours in mixture (5xSSC, 250 µg of sheared denatured salmon sperm DNA per ml, 25mM NaPO₄ (pH 6.5), 5x Denhardt's and 50% formamide).

The prehybridization mixture was removed and replaced with hybridization mixture containing 1 µg of biotin-labeled DNA probe 5'- Biotin-labeled AAAGTGTCTGGA-AAATGTACAAGTTACAGAGTTAGCACTA- 3'. The reaction was allowed to proceed at 65°C for 16 hours. Then the membrane was washed in 250 mL of 2xSSC, 0.1% (wt/vol) SDS for 5 min at room temperature (RT); 250 mL of 0.2xSSC, 0.1% SDS for 5 min at RT; and 250 mL of 0.16xSSC, 0.1% SDS for 15 min at 50°C twice. For colorimetric DNA detection, filter was washed for 1 min in 0.1 Tris-HCl (pH 7.5), 0.15 M NaCl and non specific binding sites were blocked by immersion in a 3% solution of bovine serum albumin in the same buffer for 40 min at 65°C. Streptavidin-peroxidase conjugate (1µg/mL in the same buffer) was added for 10 min with gentle agitation. The filter was washed twice in 250 mL of 0.1 M Tris HCl (pH 7.5), 0.15M NaCl for 15 min at RT. Filter was sealed in polypropylene bag with freshly prepared substrate solution containing 6 mg of 3,3 diaminobenzidine tetrahydrochloride in 10 mL of 0.05 M Tris-HCl pH 7.6 and 0.1 mL of 3% hydrogen peroxide. Development of a brown color with +ve reference was allowed to proceed for 10- 60 min in dark, then terminated by washing the filter in 20 mM Tris HCl (pH 7.5) and 0.5 mM disodium EDTA, then air dried.

*Detection of Parvovirus B19 - DNA in tissues by nested PCR

DNA was extracted from aborted products and placental tissues by using DNA high pure PCR template preparation kit (Promega,UK) as instructed by manufacture. Nested PCR assay: the nested PCR was performed according to Zerbini et al (15). In brief, 7 μ L of extracted DNA was added to PCR mix for a total volume of 50 μ L containing 5 μ L of 10 x PCR buffer, 3 μ L of 25mM MgCl₂, 2.5 U of Taq DNA polymerase (Promega, UK), 200 μ M each deoxynucleotide triphosphate (Stratagen) and 300ng of each primer. After an initial denaturation step of 5 min at 95°C, the first- round PCR amplification was performed. Then 3 μ L first- round product was transferred to a second 50- μ L PCR mix. The second - round reaction mix contained the same constituents as the first- round mix, but 300 ng of each second primer was substituted for each first primer.

The oligonucleotide primers used in the first round of amplification were 5'- CTTTAGGTATAGCCAACTGG-3' and 5'- ACACTGAGTTTACTAGTGGC-3', yielding a product of 1,112 bp. Second - round PCR was performed with primers 5'-CAAAAGCATGTGGAGTGAGG3'- and 5'-CCTTATAATGGTGCTCTGGG-3'to give a product of 104 bp. Thirty - five cycles of both first and second - round amplification were performed under the following conditions after one cycle of heating at 95°C for 5 min, 95°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min, then final extension at 72°C for 7min for one cycle. Ten - microliter samples of second -round PCR products were then analyzed by electrophoresis on 2% agarose gel. Bands were visualized by ethidium bromide staining.

*Detection of CMV - DNA in tissues by nested PCR

Two pairs of primers were used according to Krajden et al, (16). The outer primer set consisted of upstream primer JC, 5'- CATAATCTCAGGGGAGC-3', and downstream primer JD, 5'-TTGGGCTAACTATGCAGAGC-3', which generated a 305-bp. The inner primer set consisted of upstream primer JB, 5'-GAAGGCTGAGTTCTTGGTAA-3', and downstream primer JA, 5'-AGCTGCATGATGTGAGCAAG-3'. First PCR was performed by adding 5 μ L

of 10x reaction buffer containing 10 mM Tris, 50 mM KCl (pH 8.3); 3 μ L of 25 mM MgCl₂; deoxynucleoside triphosphate (dNTP) mix containing 10 mM (each) dATP, dCTP, dGTP and dTTP; 50 pmol of each primer; 2 U of Taq polymerase (Promega) and 7 μ L of sample DNA (total volume, 50 μ L). Samples were denatured at 95°C for 5 min and subjected to 30 amplification cycles as follows: denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1.5 min at 72°C, with a final extension for 7 min at 72°C in a Hybaid Express thermocycler. Three microliters of PCR products from the first PCR was reamplified by using the same amplification protocol with 50 pmol of each inner primers. PCR products of 168- bp were detected by 2% agarose gel electrophoresis. Multiple negative controls, aerosol- resistant- tips, and separate processing areas were used to minimize the risk of amplicon contamination.

Statistical analysis: Demographic, medical and laboratory information were entered concurrently into a custom- designed database. Descriptive statistics were calculated using student's t test, the X² test, crosstab / Chi-square tests. The results were expressed as mean value \pm SD. P value <0.05 and < 0.01 were considered significant.

RESULTS

The results of this study were recorded in six tables and three figures. Ten cases (10/70) were excluded from the aborted group because of hemolysis of the blood samples of some and autolysis of tissue specimens of others. Therefore only 60 miscarried women were studied in-group II. There were no risk factors for CMV or B19V infections in pregnant women of this study and we suspected exposure to their own children at home may be the only risk factor, as most infected children are asymptomatic. Further, we could not demonstrate a significantly greater risk by any maternal occupation. Table (1) showed that women in both groups were matched in maternal age.

The results of thyroid autoantibodies were recorded in Tables (2) and (3). Whereas Thyroglobulin antibodies levels were significantly higher in the aborted women than control ($p < 0.05$), there was no significant changes in the thyroid peroxidase antibodies levels between the two

groups ($P > 0.05$). Summation of the results of both thyroid antibodies were not significantly changed between the two groups. Patients were determined to be euthyroid by demonstrating normal levels of thyroid-stimulating hormone (TSH), T4 and T3. Analysis of the thyroid hormonal levels revealed that 56 women were euthyroid (mean TSH=4.1uIU/mL, mean T4 = 4.85ug/dL and mean T3=163.691 ng/dL), four women

Table 1

Age and past history of women with repeated miscarriage versus normal delivery [mean \pm SD range]

Items	Control group (I)	Study group (II)
Age (Yars)	31 \pm 3.1 (25-41)	32 \pm 2.1 (27-42)
*GA (W) at collection	39 \pm 1.2 (37-41)	12 \pm 7.8 (7-20)
No. of prior abortions	0	3.6 \pm 1.1 (2-8)
No. of primary aborters	0	50
No. of secondary aborters	0	10

*GA = gestational age in weeks

Table 2
Thyroid autoantibodies in the studied women

Anti- thyroid antibodies	Control group (n=30)		Study group (n=60)		P value
	+ve	-ve	+ve	-ve	
TG IU/mL (+ve>40IU/mL)					
Number of cases	0	30	7	53	
Mean \pm SD	-	-	64.92 \pm 50.01	21.83 \pm 11.5	<0.05
Range			44 - 150	11 - 33	S
TPO units/mL (+ ve >100 units/mL)					
Number of cases	2	28	2	58	
Mean \pm SD	228.82 \pm 150.32	31.45 \pm 15.93	400.2 \pm 196.52	17.88 \pm 10.5	NS
Range	110.2 - 346.2	1.3 - 61	132 - 667	6.5 - 75.5	

TG =Thyroglobulin antibodies
+ve = antibodies positive
S = significant

TPO = Thyroid peroxidase antibodies
- ve =antibodies negative
NS = non significant

Table 3
Antithyroid antibodies (TG +TPO) in the studied women

Groups	Antibodies Positive group	Antibodies Negative group
Control group (n = 30)		
Number (%)	2 (6.67%)	28 (93.33%)
Age (year mean \pm SD)	32 \pm 3.1	32 \pm 3.1
N of prior abortions (mean \pm SD)	0	0
Study group (n=60)		
Number (%)	9 (15%)	51 (85%)
Age (year mean \pm SD)	33 \pm 2.9	34 \pm 3.4
N of prior abortions (mean \pm SD)	4.5 \pm 1.6	3.6 \pm 0.93
P values	NS	NS

were hyperthyroid and none were hypothyroid in the recurrent miscarried group. Whereas in the control group only one woman was hyperthyroid and 29 were euthyroid (mean TSH=mean T4 = 6.44 ug/dL and mean T3= 150.02

ng/dL). These results were not statistically significant. Table (4) and figure (1) showed that detection of parvovirus DNA was significantly higher in the aborted tissues than in the placentas of the control group ($P < 0.01$). Of the women infected by B19V no one reported recent signs or symptoms consistent with parvovirus infection (fever, rash, or arthralgias), and none recalled exposure to a child with erythema infectiosum and ultrasound scans showed no evidence of hydrops fetalis. Table (5) showed the results of serum CMV IgM and tissues CMV- DNA. Figure (2) demonstrated CMV viral DNA by PCR assay. Table (6) showed that, among the aborted women, DNA amplification by PCR for parvovirus B19 was strongly positive in 19 women (31.67 %) ($P < 0.01$), of these women only 5 (26.32%) were seropositive for thyroid antibodies, 2 (10.53%) women were seropositive for CMV IgM and only one (5.26 %) demonstrated CMV - DNA in the products of conceptions. This represents statistically non-significant changes ($P > 0.05$) and revealed that there was no relationship between viral infections and thyroid autoimmunity in the studied groups. Figure (3) is a chart demonstrating the percentage of parvovirus B19 and CMV viral infections and antithyroid antibodies seropositive in both groups.

Table 4
Parvovirus B19 -DNA in sera; placentas; and products of conceptions of the studied groups

Items	Controlgroup (N=30)		Studied grou (N=60)		p value
	N	%	N	%	
Serum B19 - DNA by hybridization:					
Positive	0		0		
Negative	30	100	60	100	
Tissues B19 - DNA by PCR assay:					
Positive	2	6.67	19	31.67	
Negative	28	93.33	41	68.33	HS (p<0.001)

HS= highly significant

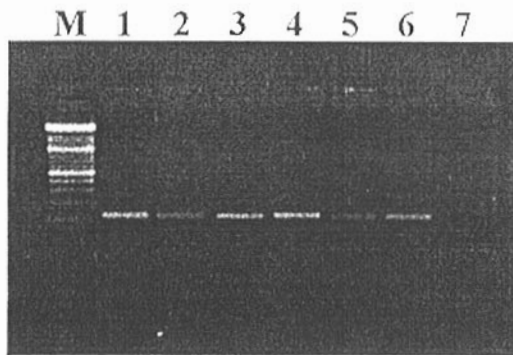


Figure 1
Agarose gel electrophoresis of PCR amplification products of parvovirus B19 DNA, M: 100 bp DNA molecular size marker, positive samples (104 bp), (lanes:1-6), negative control, lane (7).

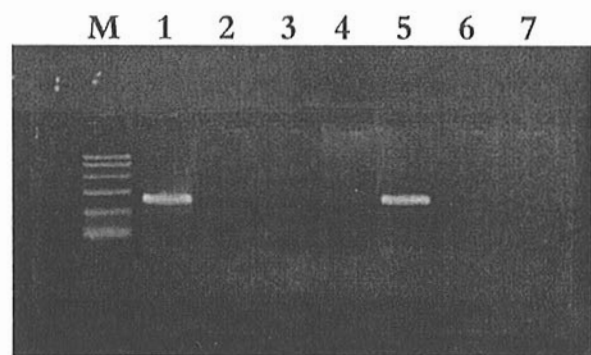


Figure 2
Agarose gel electrophoresis of PCR amplification products of Cytomegalovirus DNA, M : DNA molecular size 50 bp marker, positive samples (168 bp)(lanes: 1,5), lane (2), negative control, lanes (3,4,6,7) were negative samples.

Table 5
Repeated miscarriage versus normal delivery: Serum CMV specific IgM antibodies and CMV - DNA in the products of conceptions and placentas by PCR

Items	Controlgroup (N=30)		Studied grou (N=60)		p value
	N	%	N	%	
Serum CMV specific IgM					
Positive	1	3.33	5	8.33	>0.05 (NS)
Negative	29	96.67	55	91.67	
CMV DNA in the Tissues					
Positive	1	3.33	7	11.67	
Negative	29	96.67	53	88.33	>0.05 (NS)

DISCUSSION

Antithyroid antibodies are known to occur in apparently healthy populations and are more frequently observed in women during their reproductive years. In this study, one or both of the antithyroid antibodies (thyroid peroxidase

and thyroglobulin) were demonstrated in 15% of the patients with recurrent spontaneous abortions compared with 6.67% of the control group. This result was not statistically significant. Whereas, 85%of the miscarried women in the present study did not demonstrate thyroid antibodies in there sera, 6.67% of the control group, that were anti-

Figure 6
The frequency of CMV infection and thyroid autoantibodies in women with evidence of B19V infection by PCR

	Control group Placenta B19V PCR		Studied group Products of conception B19V PCR		p value
	+ve (n=2)	-ve (n=28)	+ve (n=19)	-ve (n=41)	
Serum CMV IgM					
+ve	0	1 (3.56%)	2 (10.53%)	3 (7.32%)	NS
-ve	2 100%	27 (96.44%)	17 (89.47%)	38 (92.68%)	
Tissues CMV PCR:					
+ve	0	1 (3.56%)	1 (5.26%)	6 (14.63%)	NS
-ve	2 100%	27 (96.44%)	18 (94.74%)	35 (85.37%)	
Thyroid autoantibodies:					
+ve	0	2 (7.14%)	5 (26.32%)	4 (9.76%)	NS
-ve	2 (100%)	26 (92.86%)	14 (73.68%)	37 (90.24%)	
total	2 (6.67%)	28 (93.33%)	19 (31.67%)	41 (68.33%)	

+ve = women demonstrated B19 V DNA in the placenta and products of conceptions

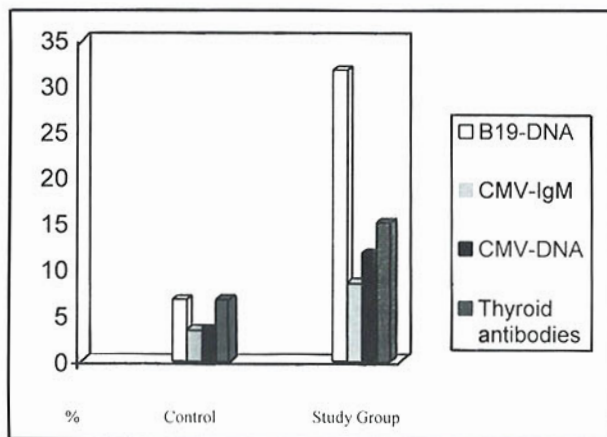


Figure 3
The percentage of positive parvovirus B19 - DNA and CMV - DNA in tissues , CMV- IgM seropositive and antithyroid antibodies seropositive results in the studied women.

thyroid seropositive, had delivered healthy babies. In spite thyroglobulin antibody levels were significantly higher in women with recurrent abortion than control. Stagnaro et al (5) reported that the individual levels of antithyroid peroxidase and antithyroglobulin autoantibodies were similarly related to the increased rate of miscarriage, with no evidence of autoantibody specificity. Therefore, we suggested that antithyroid antibodies might serve as independent factor for the risk of repeated pregnancy loss.

On the contrary, Pratt et al. (17) reported a higher incidence of antithyroid antibodies in women with a history of three or more consecutive pregnancy losses as compared with a control population (31% versus 19%). Singh et al (18) also found that thyroid antibodies might be useful markers for identifying women at risk of clinical miscarriage. In the antibody-positive group they found that there was 32 % clinical miscarriage rate in comparison to 16% in the antibody-negative group. They reported a strong association between antithyroid autoantibodies and spontaneous abortion.

Stagnaro - Green et al (19) supported the concept that the basic immunologic defect in autoimmune diseases probably lies at the T-lymphocyte level. Pratt et al. (17) supported the hypothesis that antithyroid antibodies may serve as peripheral markers for abnormal T-cell function that, in turn, may be responsible for pregnancy loss and demonstrated that detection of thyroid antibodies before conception carries an increased risk of pregnancy loss. It is then tempting to speculate that abnormal T-lymphocyte function, and not abnormal autoantibodies per se, is responsible for pregnancy loss. Under such a concept, they reported that organ-specific autoantibody abnormalities, such as thyroid antibodies and non organ-specific autoantibodies, can be expected to have similar prognostic significance for impending pregnancy loss. Muller et al (3) concluded that successful treatment of autoantibody-associated pregnancy loss should concentrate on attempts to modulate T-cell function rather than the customary

treatment options of antibody suppression and/or modulation of antibody effects.

Whereas B19 viral DNA was detected in fetal and placental tissues, by PCR, nearly in one third of the miscarried women and 6.67% of the control group, viral DNA was not detected directly in sera by dot blot hybridization in the two groups. This finding could be explained on the basis of less sensitivity of hybridization technique than PCR. On the other hand it may be attributed to less viral concentrations in peripheral blood. In fact, the nested PCR can disclose a single copy of B19 -DNA (20), while dot blot hybridization technique have been shown to detect more than 104 viral particles (21). In the present study, the total number of tested sera was 90 samples which are considered less than that tested by Mori et al (22) and Yoto et al (14) who used hybridization technique for detection of B19V -DNA in the serum and found positive reactions in 15/71969 (0.2%) and 48/41895 (0.9%) respectively. Polymerase chain reaction-based assay was shown to be a more sensitive and accurate means of diagnosing B19 V infection. The problem with polymerase chain reaction-based methods is not their lack of sensitivity but rather their extraordinary sensitivity (23).

Gratacos et al (24) and Harger et al (9) reported that the overall incidence of fetal loss linked to parvovirus B19 infection was 1.66 % and 8.3 % of B19- IgM-positive pregnant women respectively. The figures considerably lower than that reported in the present study. Gratacos et al (24) findings provided evidence that parvovirus infection during pregnancy is a rare cause of fetal morbidity and mortality and they suggested that adverse outcomes are infrequent even in a population with a high incidence of infection. On the contrary Jordan (25) reported that B19V infection of pregnant women have been associated with poor fetal outcomes, including fetal anemia, nonimmune hydrops, spontaneous abortion, and stillbirth. However, transplacental transmission rate of B19V has been estimated to be about 33 %, indicating that this virus could be more fetotropic and fetotoxic than either rubella or cytomegalovirus. Therefore, it is important for the physician to have accurate clinical data regarding the immune status of a pregnant woman when an exposure to or infection by B19V is suspected. Adults may have no clinical evidence of disease even though serologic studies, IgM and IgG antibodies would indicate recent infection. Jordan (23) concluded that fetuses should be monitored for up to 14 weeks after maternal exposure to B19V infection.

Cytomegalovirus infection during pregnancy is far more complex than other infections, such as rubella and toxoplasmosis, which occur only with primary infections, because of the ability of this virus to become reactivated frequently during the childbearing years and to be transmitted to the fetus in spite of maternal immunity (7). Of all investigate miscarrying women in our study the percentage of seropositive and seronegative mothers did not differ significantly. This result was in agreement with that of Stango (7).

Early diagnosis of CMV leads to dramatically better prognosis; a hypothesis that has to be examined (7).

However, this still leaves the problem that there is no specific and effective therapy for congenital CMV infection that can be administered during pregnancy. This leads to the phenomenon that most clinicians do not screen at all. In order to overcome these problems a step-wise screening of CMV to avoid a false positive result is needed. This is necessary because a mother expressing CMV-IgM does not necessarily transmit the virus to the child as observed in our results. Positive IgM anti-CMV results may furthermore be false-reactions with other substances, such as rheumatoid factor, or nonspecific reactions. Baumann et al (8) reported a low specificity of IgM anti-CMV test. However, in a screening program false positive results can be rather accepted than false negative results, since the positive serological result has to be confirmed by a specific and sensitive methods as virus isolation by shell vial cell culture assay and by PCR for CMV - DNA detection (26). In order to verify whether the serological screening of mothers in the present study really detected transplacental transmission and hence detection of congenitally infected fetuses, CMV specific IgM levels were compared with screening for CMV - DNA in fetal and placental tissues. We found only two out of the 5-CMV - IgM positive miscarried women demonstrated viral DNA in the products of conceptions. So that not all CMV - IgM seropositive miscarried women transmitted the virus to their fetuses.

Although the etiology of autoimmune diseases is unknown, there are some clues as regards the predisposing factors: genetic factors, certain HLA class I and II alleles or invading microorganisms may be involved in the early stage of the disease and T-cells are often involved in the etiology and pathogenic process, in fact the etiology is likely to be multifactorial (27). In the current study we did not find any significant association between thyroid autoimmunity and viral infections in women with unexplained recurrent miscarriage.

We concluded that parvovirus B19 infection represent nearly one third of the etiology of unexplained recurrent abortions in the present study. Therefore it should be included in routine screening of cases of recurrent abortion. However, in the present study, the detection of cytomegalovirus infection in aborted women does not support a role for CMV infection as a major abortion related factor. Thyroid autoimmunity might serve an independent pathogenic risk factor for repeated pregnancy loss. Testing for IgM anti-cytomegalovirus and antithyroid antibodies should be restricted to certain patients with unexplained recurrent pregnancy loss.

We recommend using PCR assay of placenta or fetal blood /tissue samples for screening of potential B19V or CMV infections in utero.

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