Original Article

An association between *waddlia chondrophila* and human miscarriage; identifying it in placenta or genital tract

Saeed Harirzadeh [1], Farshid Kafilzadeh [1]*, Hengameh Zandi [2]

[1] Department of Biology, Jahrom Branch, Islamic Azad University, Jahrom, Iran. [2] Department of Microbiology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Correspondence: Farshid Kafilzadeh, Department of Biology, Jahrom Branch, Islamic Azad University, Jahrom, Iran. Email: f.kafilzadeh@gmail.com

ABSTRACT

Waddlia chondrophila is an intracellular bacterium that causes miscarriage in human and cattle. Overall, 50% of cases of miscarriage and recurrent miscarriage is unknown. Obviously, miscarriage for an unknown reason can be influenced by an unknown infectious agent. Researches shows that at least 2 percent of recurrent miscarriages are also directly related to infectious agents. However, no information is available in respect to the presence of this bacterium in Yazd. Due to the low sensitivity of cell culture to recover such an obligate, molecular-based diagnostic approaches are necessitated. This research investigates the development of a quantitative SYBR Green assay which targets the *recA* gene of *W. chondrophila*.

Keywords: *Waddlia chondrophila*, miscarriage, intracellular bacteria.

Introduction

A miscarriage is defined as any loss before 20th weeks of pregnancy, typically these losses taking place before the thirteenth week [1]. Just about 25% of pregnant women will experience at least 1 miscarriage case [2, 3]. Miscarriage occurs for a variety of medical reasons, many of which are not under control, although the number of diagnostic tests and therapeutic interventions in the control of miscarriage have grown remarkably, a cause is identified in only 50% of cases [4, 5].

Miscarriages can happen for a variety of medical reasons, many of which aren't within a person's control. Understanding these risk factors and symptoms can help you be more aware of future possible events and get any medical assistance or treatment you might need.

Waddlia chondrophila belongs to the Chlamydiales order. It was originally identified in aborted tissue samples which has been recently associated with miscarriage in human [6, 7]. *Waddlia chondrophila* is an intracellular bacterium, which do not grow on routine culture media, represent possible agents of miscarriage with unexplained etiology [8]. Studies shows that *waddlia* can infect human cell lines and primary macrophages [9]. Cell culture technique is one of the diagnostic methods which is not suitable in the research since the *Waddlia chondrophila* do not grow on routine culture media.

A real-time PCR method based on the 16sRNA gene has recently been developed [10]. This technique can perform in several ways that SYBR Green method is the simplest one. The SYBR Green-PCR method in comparison with the Taqman method has a simpler design and a lower cost [11], while has a more accuracy and better result [12].

Access this article online	
Website: www.japer.in	**E-ISSN:** 2249-3379

How to cite this article: Saeed Harirzadeh, Farshid Kafilzadeh, Hengameh Zandi. An association between *waddlia chondrophila* and human miscarriage; identifying it in placenta or genital tract. J Adv Pharm Edu Res 2020;10(S4):83-87. Source of Support: Nil, Conflict of Interest: None declared.

Materials and Method

Sample Collection

In this prospective observational study, 100 clinical specimens were collected from vaginal swabs, cervix, gynecological blood and fetal tissue. All clinical samples were collected from the 54 healthy women. Vaginal swabs are performed using sterile Dacron swabs and speculum. Fetal specimens are stored in PBS buffer medium and in transitional medium for subsequent steps.

Anti-Chlamydia antibody titer

Unfortunately, there is no commercial kit for this bacterium, so since it is in the Chlamydia family, a commercial ELISA kit was used to track Chlamydia.

Positive control

In order to construct positive control, the gene sequence was designed by Gene Fanavaran Company (Tehran, Iran). The gene was also cloned into the PUC57 vector by the same company. In the next step, this vector was transformed in *Escherichia coli* and then, this plasmid was extracted by the plasmid extraction kit (Yekta Tajhiz Azma, Tehran, Iran) and used as a positive control.

Molecular identification

Bacterial identification was confirmed by Real-time PCR. The sequences of the specific primers were Forward primer: CGGCTACTGTTCTGTATC, Reverse primer: GCGTATAACCCTTTGCTTA and Probe: 5-AGTCCACCTGAACCTGATGC-3.

DNA was extracted using Geneaid Extraction Kit according to the kit manufacturer's instructions. Each PCR reaction includes, 1.0 µL of template DNA, 12.5 µL of CinnaGen Real-time Master Mix, 2.0 µL of each primer, and 9.5 µL of ddH2O. The PCR reaction was carried out for 35 cycles in an Eppendorf MasterCycle Gradient thermocycler (Eppendorf, Hamburg, Germany) with the fallowing program, initial denaturation at 95°C for 5 min, denaturation at 95°C for 60 s, annealing at 57°C for 30 s, extension at 72°C for 40 s and a final extension at 72°C for 5 min. To analyze all samples (aborted and controls) by syber Green Real time PCR, we used 5 µl of each DNA, 1 µl of each primer, 12.5 µl of master mix, 1 µl of each *Waddlia chondrophila* primer, 0.5 µl *Waddlia chondrophila* Probe, 0.5 µl internal control probe and 2.5 µl DEPC water. All samples were tested in duplicate for three different assays.

Determination of syber Green Real-time PCR Reaction Sensitivity of Bacteria

The extracted DNA was determined by the positive control of the bacterium and then from the concentration of 500 ng Microliters with a dilution factor of 10.1 were prepared for 5 consecutive dilutions. DNA dilutions respectively. We designated 1: 10-1: 100-1: 1000-1: 10000-1: 100000 as an example to determine sensitivity. The test was repeated three times.

Specificity of syber Green Real-time PCR Reaction of Bacteria

To investigate the specificity of the designed primer reaction, the specificity test was performed with 3 sexually transmitted bacteria. The genomic DNA of three bacteria was extracted from DNA samples of Chlamydia, Mycoplasma and Legionella and real time PCR was performed on them. This test was performed 3 times.

Result

IgG and IgM ELISA

The total sample size in this study was 100, of which 54 were controls and 46 were women with history of recurrent or sudden miscarriage. Serum samples of women were used for the ELISA kit of Euroimmun to determine the titers or the presence of anti-Chlamydia antibody (Table 1, Fig 1).

Table 1 - Number of people with antibody titers in the control and target groups

Type of Anti-body	control group	Target group
IgG	1positive sample	6 positive samples
IgM	Lacking IgM	1 positive sample

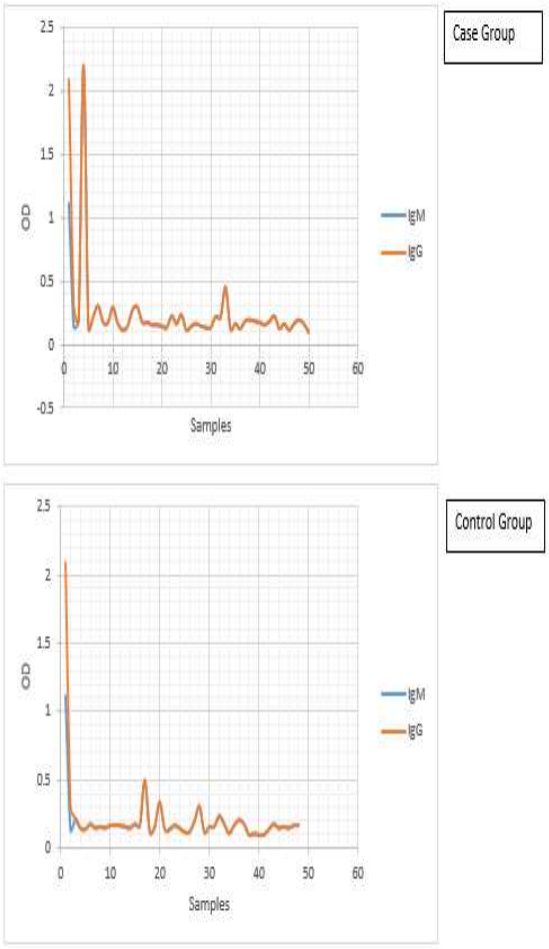

Figure 1- ELISA results case and control samples

DNA extraction

The patient's DNA samples were extracted and the results were observed on electrophoresis agarose gel (Fig 2).

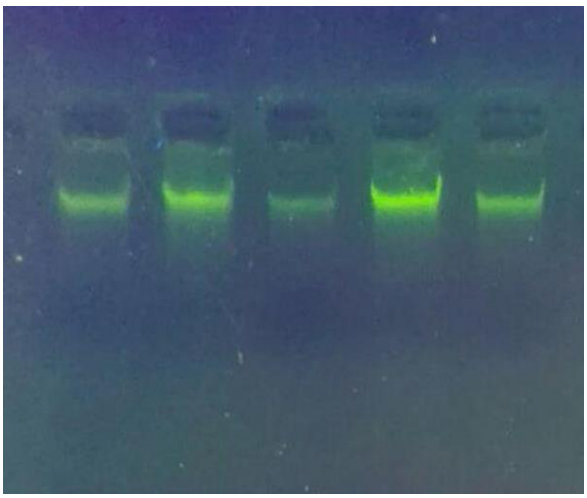

Figure 2- DNA Extraction on Gel

Cloning results of the Waddlia bacterium gene within the PUC57 vector

Cloning of the PCR product in the PUC57 vector was carried out to construct a positive control by Korea Corporation and recombinant vector was prepared. We inserted the recombinant vector into Escherichia coli susceptible to transformation. Single colonization culture was performed to identify the recombinant vector and then plasmid extraction was performed. To ensure the accuracy of plasmid extraction, sample was taken on a gel and electrophoresis was performed and the results are in the following form (Fig 3).

Figure 3- Recombinant plasmid extraction

Result of PCR test using primer designed

PUC57 was then performed using dedicated primers according to appropriate thermal instructions. The results were observed on gel electrophoresis with 100 bp marker (Fig 4).

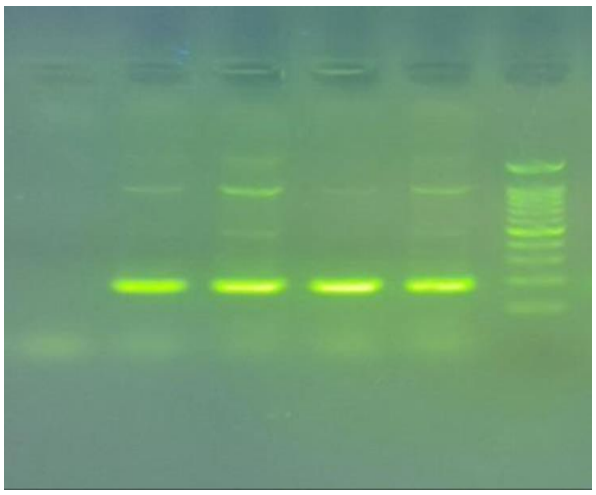

Figure 4- Gel electrophoresis of PCR of RecA Waddlia. Column No. 1 Marker 100 bp, Columns 5 -2 Positive Samples. Column-6 Negative Control

Results of syber Green Real-time PCR Reaction Regulation for Bacteria

In the syber Green test shown in Figure 6-3, there were no positive samples (Fig 5).

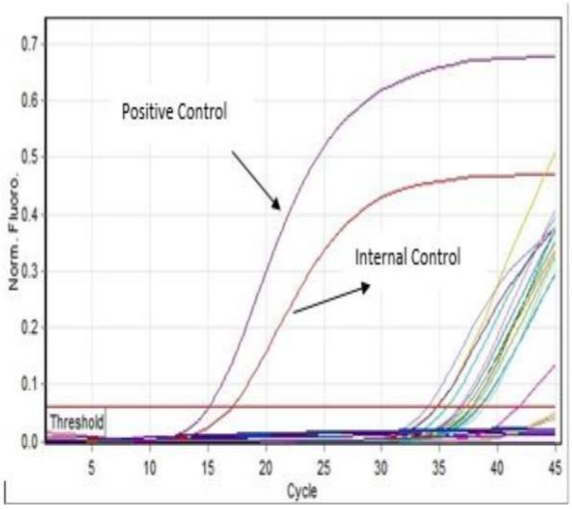

Figure 5- Results of the Taqman reaction of *Wadelia* bacteria

Syber Green Real Time PCR Sensitivity Test for Bacteria

The results of the sensitivity reaction are outlined below (Fig 6 ، Table 2). Results above CT higher than 35 were considered negative. The best result for use in the syber Green reaction was 5 ng / ml.

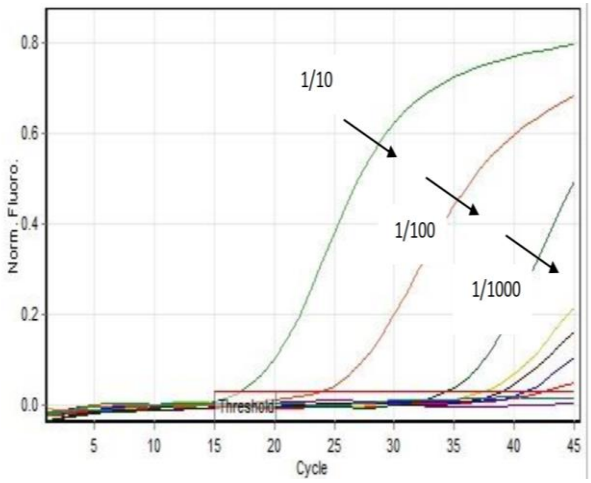

Figure 6- The results of the sensitivity reaction of syber Green

Table 2- Sensitivity of syber Green reaction of bacteria

No	Name	CT
1	NTC	42.72
1	1:10000-pc	37.73
2	1:100000-pc	39.20
3	1:1000-pc	.5753
4	1:100-pc	24.24
5	1:10-pc	17.31

Syber Green Real Time PCR Specificity Test for Bacteria

To determine the specificity of the test, DNA samples from Chlamydia, Mycoplasma and Legionella bacteria were used, which primers did not induce nonspecific reactions with any of the above (Fig 7).

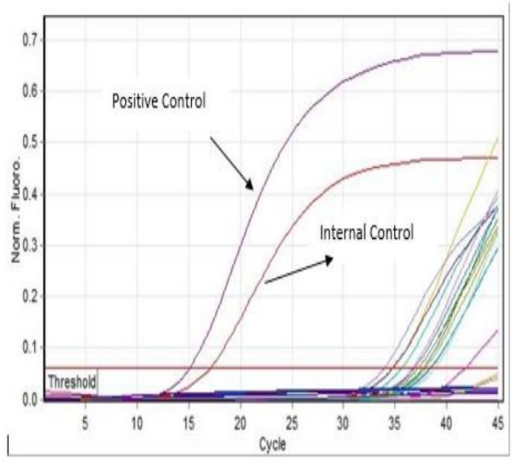

Figure 7- SYBER Green reaction specificity results

Reproducibility results of Syber Green bacterial reaction

You can see the reaction results in the figure. The results showed a good correlation between the results of the three runs in three days (Fig 8).

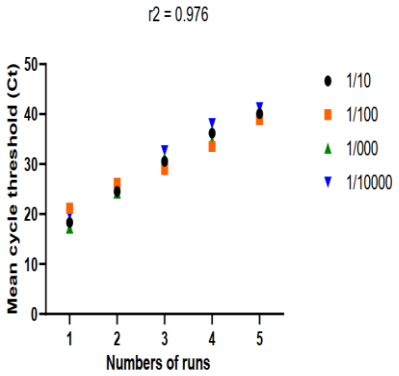

Figure 8- a good correlation between the results of the three runs in three days

Discussion

Waddlia chondrophila, a Chlamydia-related bacterium first indicated in samples of bovine abortion tissues, has been correlated with miscarriages in human [13].

Miscarriage and recurrent miscarriage are the most complicated and life-altering experience. This leads to clinical consequences such as damage to intrauterine devices and recurrent urinary tract infections. In addition, miscarriage has an emotional effect and can also lead to psychological disorders in women [2].

During the last two decades, researches on diagnosis of infectious disease are growing day by day which has greater impact on infectious disease control.

Nucleic Acids base methods for Multiple Samples with High sensitivity is economically viable.

In recent years, development of molecular diagnosis methods has been greatly influenced by several factors such as: extensive developments in rapid isolation of Nucleic Acids and advances computer usage in Analysis of nucleic acids technology.

The diagnosis of *Waddlia chondrophila* is clinically principal. Transmission method of this bacterium is still unknown, components are found predominantly in biological specimens such as serum, feces, and so on. The available diagnosis methods includes phenotypic analysis methods in culture medium, serological analysis and immunofluorescence. However, these microorganisms are difficult to isolate from the medium culture because of the Low concentration and resistance to the media.

The purpose of this research was to determine *W. chondrophila* in placenta or genital tract of women who had miscarriages by using Real Time PCR method. There is a strong association between the presence of *W. chondrophila*–specific IgG antibodies and early fetal loss [6].

Researchers investigated the development of a quantitative SYBR Green real-time PCR assay targeting the recA gene of W. chondrophila on veterinary samples. The results showed that this bacterium is an abortigenic agent in cattle [14].

In addition, Waddlia chondrophila induces systemic infection, organ pathology, and elicits Th1-associated humoral immunity in a murine model of genital infection [14].

Researchers confirmed an association between antibodies against *W. chondrophila* and human miscarriage and identified this organism in placenta or genital tract of women who had miscarriages. These results suggest a possible role of *W. chondrophila* infection in miscarriage [15].

In this study, W. chondrophila was optimized in terms of sensitivity and specificity. This method allowed the detection of *W. chondrophila* DNA. According to available evidence, this bacterium is an important contributor in miscarriage. In this study, we investigated 46 specimens from vaginal and cervical swabs and blood of women and fetal tissue by syber Real time PCR method. However, *W. chondrophila* DNA has not been detected in any samples.

According to the results of our study, we'll able to design a Real time PCR kit with high sensitivity and specificity to detect easily chlamydial infections in diagnostic Medicine laboratories.

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