DETECTION OF BORRELIA BURGDORFERI SPECIFIC DNA IN TISSUES BY PCR TECHNOLOGY

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SUMMARY

The amplification of DNA by polymerase chain reaction can be used for detection of *Borrelia burgdorferi (Bb)* genome in fresh tissues and also in formalin-fixed and paraffin embedded tissues. The method is a multistep procedure From a histological section 4-8 microns thick enough DNA can be extracted for amplification. The specific sequences of Bb DNA of less than 100 nucleotides are preferred. The amplification is performed in a thermocycler with tube cups heating system. It is concluded that the detection of *Bb* genome in tissues by PCR is a powerful diagnostic tool, however for economic reasons not suitable as a routine procedure.

KEY WORDS

Borrelia burgdorferi, detection, polymerase chain reaction, tissues, skin

INTRODUCTION

Recently the amplification of deoxyribonucleic acid (DNA) by polymerase chain reaction (PCR) was introduced as a method (1) for the detection of *Borrelia burgdorferi (Bb)* genome in cerebrospinal and synovial fluids, serum, urine and fresh tissues(2-10).Some authors described the PCR application for specific DNA detection also in formalin-fixed and paraffin-embedded tissues(11,12). *Bb* can be isolated from early skin manifestations (erythema chronicum migrans, ECM) as well as from late skin lesions (acrodermatitis chronica atrophicans, ACA).The detection of Bb genome in formalin-fixed and paraffin-embedded skin biopsies is a very sensitive method and therefore of great interest to define better skin lesions associated with *Bb* infection. The method represents a new and powerful tool for the diagnosis of Borrelia induced infections.

METHOD

A multistep procedure for the detection of the *Bb* genome in formalin fixed and paraffin embedded skin specimens is described

Treatment of tissues

The skin biopsies are usually just small fragments of tissue and therefore the procedure to use such small quantities of tissue for a DNA molecular analysis is rather complicated. However the advantage is that 1. the same biopsy may serve for morphological examination, 2. a histological section only a few microns thick may be used for the analysis and 3. materials stored from previous pathohistological examinations can be used, so that there is no need for a new biopsy.

For DNA analysis the time between the biopsy and the fixation is not essential, as DNA can be analyzed also when a fixation is performed after hours or even days. The PCR method can actually be used also when the DNA is partially degraded.

Most of the fixatives routinely used in histopathology allow an excellent DNA preservation. Tissues fixed in 10 % buffered formalin, ethanol, acetone, paraformaldehyde give a DNA sufficiently conserved for the PCR analysis, but Bouin's, Zenker's and B5 fixatives are usually less adequate.

From a histological section 4-8 microns thick enough DNA can be extracted for one or more amplifications and if the tissue fragment is very small more sections can be cut from the paraffin block. The sections can be laid on a histological slide and then scraped away with a sterile scalpel for the DNA extraction, or better put directly in a sterile tube. The section should not be touched with hands or with anything that is not completely clean or sterile. A cross-contamination among samples can occur but in our experience the cleaning of the area between a sample and the following one is sufficient to prevent it. The microtome blade does not need to be changed after each case: the trimming of the block, cutting few sections before the section for the DNA extraction, is adequate to clean the blade.

This type of cross-contamination is avoided using as negative control a paraffin block without any tissue that we cut with the tissue samples and then process together in all the steps.

Before the DNA extraction it is advisable to look at a stained histological section to see the presence of necrotic or hemorhagic areas. Blood degradation products can inhibite the PCR amplification process.

Extraction of DNA from tissues

The first step is the deparaffinization of the sections: in case that the tissue section are on a slide, the deparaffinization can be performed as usually in histopathology and the tissues scraped and put in a tube after the deparaffinization step. When the sections are not on the slide but in a 1.5 ml tube they are treated with xylene, absolute alcohol and 70% alcohol for some minutes. After each treatment a rapid centrifugation is performed, the supernatant discarded, and the tissues are then air dried.

After this common deparaffinization step many methods are suggested for the DNA extraction (13). The method we prefer gives a quite pure DNA free of proteins which could interfere with the subsequent steps. Briefly the tissues are digested over night with proteinase K (500 mg/ml) in buffer Tris-HCl 50 mM pH 7.5, EDTA 1 mM, Tween 20 0.5%. An extraction in phenol chloroform and precipitation in absolute ethanol is performed. We suggest a long time of precipitation, over weekend, to recover most of degraded DNA.

This is a time consuming procedure, but more constant results are obtained, particularly in tissues in which the sequence to be analyzed is scarce, like the Borrelia genome in skin lesions.

Amplification of DNA

First a specific sequence of Borrelia DNA should be chosen. The choice depends on many factors, first of all on the purpose of the analysis. In the case that different strains are to be detected, a characteristic sequence for each strain is to be used (3,14). Anyway for the diagnosis of the infection with *Bb* or their persistence in tissues, a sequence which is constant in every Borrelia strain can be applied. The sequence to be amplified should be short enough to increase the efficiency of the method, because of a possible degradation of the DNA and of the scarcity of the sequence investigated compared to the DNA of the host. We prefer a sequence of less than 100 nucleotides.

We synthesized 3 oligonucleotides from the flagellin gene sequence (2):

BFl: 5'CTGTTGAGCTCCTTCCTGTTG3';

BF2: 5'TCAGGCTGCACCGGTTCAAGAGGGTGTT3';

BF3: 5'TTCTCTGGTGAGGGAGCTCAAC3'.

BFl and BF3 are used as primers for the PCR amplification. They spun a DNA fragment of 75 bases. BF2 represents a sequence internal to the amplification fragment between BF1 and BF3. The amplification was performed in a solution containing, for 100 µl 0.4-1.0 µm of extracted DNA in 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 20 µM for each dNTP, 0.15 µM of each primer and 2.5 U of Taq polymerase every 100 µl of final solution. We perform the amplification with the same conditions also in 50 and 20 µl.

The amplification is performed in a thermocycler with tube caps heating system. After an initial denaturation at 94°C for 3 min, 5 cycles at 94°C / 1 min, 55°C /1 min, 72°C /1 min and 35 or 65 cycles at 94°C /30 sec, 55°C /30 sec, 72°C /30 sec were performed. We perform many cycles of 30 sec for step with a very short amplified DNA fragment because the DNA is partially degraded and the specific Borrelia DNA is often scarce in comparison with the host tissue DNA. We avoid the nested PCR, performed by many authors (3,6, 7,9), because a repeated PCR procedure in the same sample increase enormously the possibility of carry-over of material among samples with false positive results.

Controls

We perform negative controls along all the steps of the method using histological sections from a paraffin block not containing tissues. Positive controls should also be performed but with a very small quantity of specific DNA template, to avoid a dangerous source of carry-over and to check the sensitivity of the procedure. An important control concerns the accessibility of DNA in the skin tissues in exam, for this purpose we perform a parallel amplification of a host genome DNA, that show the level of degradation of DNA in the sample and makes the samples comparable.

The prevention of false positive results should be particularly careful following the rules recommended by Kwok and Higuchi (15).

Analysis of results

For the analysis of the results 10 µl of the amplification products are electrophoresed in a 2.5% agarose minigel and then blotted on a nylon membrane for a Southern-blot. The oligonucleotide internal to the amplified sequence (BF2) is used as probe. We label the probe with 32P, but non-isotopic reporter molecules can also be used.

A Southern-blot is performed because the amplification of a short fragment of DNA (75 bases) can not always be easily recognized on a agarose gel, as the fluorescent dye of the gel does not bind easily to short fragment of DNA, and the positive band can be confused with specific amplification band.

CONCLUSIONS

Detection of the *Bb* genome in tissues by the PCR method can be regarded as a powerful diagnostic tool. This method is potentially helpful in solving the still not fully understood problems concerning the late infection with *Bb* in general as well concerning the late skin manifestations. It makes possible also the investigation of the mRNA expression of Bb in skin and other tissues, even in paraffin embedded material (16).

Unluckily the PCR method is at present quite expensive and complicated, and for these reasons not suitable as a routine procedure. It should be however used in all instances were the diagnosis is not clear or doubtful and for systemic clinical studies aimed at solving certain pathogenetic problems.

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