Molecular diagnosis of bloodstream infections caused by non-cultivable bacteria

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Abstract

Bloodstream infections are an important cause of morbidity and mortality in patients. Blood culture is clearly the most important diagnostic procedure for identifying micro-organisms involved in bloodstream infections except when the patient has previously received antibiotics or in the presence of slow-growing or intracellular micro-organisms. Detection of micro-organisms, mainly in blood, using pathogen-specific or broad-range PCR assays is promising. However, it is very important to emphasise that the interpretation of this molecular tool is critical because of the risk of interfering contamination, underlining the necessity to interpret the results obtained with caution. Presently, due to more widely available data and to rapid advances in biotechnology, two significant improvements allow new perspectives for molecular diagnosis. Indeed, the complete sequences of genomes have provided an important source of gene sequences for PCR-based assays. In addition, the development of real-time PCR offers several advantages in comparison to conventional PCR, including speed, simplicity, quantitative capability and low risk of contamination. Herein, we review the usefulness of molecular diagnosis of highly fastidious micro-organisms in the context of three different bloodstream infections: systemic diseases (rickettsiosis, Q fever, bartonellosis, Whipple’s disease), blood-culture-negative endocarditis and bioterrorism attack.

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1. Introduction

Bloodstream infections are an important cause of morbidity and mortality in patients [1]. Blood culture is clearly the most important diagnostic procedure for identifying micro-organisms involved in bloodstream infections. Ideally, blood samples should be taken immediately prior to the start of empirical antimicrobial treatment. However, the blood culture is slow and insufficiently sensitive when the patient has previously received antibiotics or in the presence of slow-growing or intracellular micro-organisms. For example, blood cultures miss highly fastidious micro-organisms that are responsible for blood-culture-negative endocarditis (BCNE), such as Bartonella spp., Coxiella burnetii, Mycoplasma spp., Chlamydia spp. or Tropheryma whippelii.

Early antimicrobial treatment has an influence on the outcome of patients. When slow-growing micro-organisms are suspected, empirical therapy will have been identified long before culture results become available [1]. If patients deteriorate or do not respond to initial empirical therapy, physicians are likely to make an empirical change in therapy before culture results are available. Thus, faster detection of bloodstream infections permits earlier implementation of adequate antimicrobial treatment, thereby reducing morbidity and mortality.

Recent terrorist attacks have increased concern about the use of biological agents by rogue countries or terrorist groups. In this specific context, there is an urgent need to clearly identify the micro-organisms involved. Most bioterrorism bacteria are highly fastidious, and for culture, at least an L3 biosafety level laboratory is required. Thus, improvement in molecular diagnosis is essential to manage terrorist attacks.

Detection of micro-organisms, mainly in blood, using pathogen-specific or broad-range PCR assays is promising [1]. However, it is very important to underline that the
interpretation of the results of this molecular tool is difficult. Problems arise from the detection of DNA rather than living pathogens, the risk of interfering contamination, and the lack of a gold standard.

The advent of molecular techniques opens a new area. Herein, we examine the usefulness of molecular diagnosis of highly fastidious micro-organisms in the context of three different bloodstream infections: systemic diseases (rickettsiosis, Q fever, bartonellosis, Whipple’s disease), bloodstream-negative endocarditis and bioterrorism attack.

2. General rules of molecular diagnosis on bloodstream infections

2.1. Choice of samples

PCR assays can be performed on sera collected in a dry tube or on blood collected in an anticoagulant (EDTA) tube. In case of suspicion of BCNE or vascular infection, PCR assays can be performed on fresh samples from cardiac valves or vascular biopsies. PCR can also be performed on paraffin-embedded biopsies but this approach may be less sensitive [2].

2.2. Choice of primers and PCR assays

Currently, the choice of technology depends on the problem (Table 1) [3]. In the absence of specific clinical suspicion, broad-range PCR, using primers targeting the 16S rRNA gene, the 23S rRNA gene, and the rpoB gene, are particularly suitable as they are ubiquitous to all bacteria [3]. The main disadvantage of this approach is that amplification should be systematically followed by sequencing or hybridisation. A circumstance in which pathogen-specific PCR is useful is when sensitive and rapid diagnosis is needed because life-threatening infection or bioterrorism attack are suspected. The increasing number of bacterial genomes sequenced (428) allows a rational in silico approach to choosing DNA targets such as specific, repeated sequences for diagnosis [4]. To overcome this problem, multiplex PCR assays have been designed in which several pathogen-specific PCRs are done simultaneously.

A significant advance in PCR technology is quantitative real-time PCR in a closed system, in which amplification and detection of amplified products are coupled in a single vessel. This speedy process eliminates the need for post-amplification processing, conventionally needed for amplicon detection, and allows for measurements of several products. Two strategies exist for real-time monitoring: the use of fluorescent DNA intercalating dyes, which bind non-specifically to double-stranded DNA generated during amplification; and the use of DNA probes with specific annealing within the target-amplified region. Regardless of the format chosen, the internal probes emit a fluorescent signal during each amplification cycle only in the presence of targeted sequences, with signal intensity increasing in proportion to the amounts of amplified products generated. This technique presents several advantages over conventional PCR, including speed, simplicity, reproducibility, quantitative capacity and low-risk contamination.

Usually, nested-PCR should be avoided except in very specific conditions such as suicide PCR.

2.3. Validation of PCR assays

To validate PCR assays, the inclusion of positive and negative controls in each PCR run is critical. All the controls must be correct to interpret the results.

For broad-range PCR, positive controls that may be confused with the causative micro-organism should be avoided, as carry-over contamination is common. For BCNE, the use of DNA from a micro-organism that is very unlikely to cause infective endocarditis (IE) should be preferred. For specific PCR, it is appropriate to take infrequent pathogenic or non-pathogenic micro-organisms, such as Rickettsia montanensis for rickettsiosis [3].

The use of negative controls, processed from DNA extraction to PCR run in parallel to the test samples, is necessary to detect PCR contamination. Samples should be separated every five samples by a negative control including water, PCR mix run, and DNA extracted from human control tissue or arthropod free of infection with rickettsiosis [3].

2.4. Interpretation of broad-range PCR assays

For broad-range PCR, each positive amplicon must be systematically sequenced for an accurate identification of the causative micro-organism as some sequences usually result from contamination. It is generally easy to recognise such contaminant DNA, which is usually from micro-organisms commonly present in water or in reagents (Pseudomonas spp.,

<table>
<thead>
<tr>
<th>Queries DNA targets</th>
<th>Queries DNA targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>General and species identification</td>
<td>Broad-range PCR targeting the 16S rRNA, 23S rRNA, rpoB</td>
</tr>
<tr>
<td>Species identification</td>
<td>rpoB, gltA, SOD</td>
</tr>
<tr>
<td>Specific targets</td>
<td>Outer membrane protein (omp) gene, specific repeated sequences</td>
</tr>
<tr>
<td>Epidemiology</td>
<td>ITS 16S–23S</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Queries DNA targets</th>
<th>Queries DNA targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic spacer (ITS)</td>
<td>Tandem repeats</td>
</tr>
<tr>
<td>Variable number tandem repeat (VNTR)</td>
<td>House keeping genes such as rpoB, gltA, SOD</td>
</tr>
<tr>
<td>Multi locus sequence typing (MLST)</td>
<td>DNA spacer</td>
</tr>
</tbody>
</table>

rpoB, RNA polymerase beta-subunit-encoding gene; gltA, citrate synthase; SOD, superoxide dismutase.
Escherichia coli). In addition, a similar sequence found in the same PCR run in a sample from another patient may also result from contamination. An original sequence, which is detected for the first time in a laboratory, usually corresponds to a true positive. When a result has a low predictive value, a positive PCR targeting a second gene is critical to confirm the aetiological diagnosis.

2.5. Source of contamination and solutions

The main problem with PCR is the risk of laboratory contamination in addition to contamination introduced when the sample is obtained (Fig. 1). The risk is present at several steps of the PCR procedure, from the taking of the samples, the isolation of the DNA, and the actual performance of the PCR assays [1,3]. Even the kits used for isolation of DNA from the samples and the PCR reagents can be contaminated. Contamination of columns used for DNA extraction with Legionella DNA has been described twice [1].

3. Fastidious micro-organisms and systemic diseases

3.1. Rickettsiosis

3.1.1. Clinical context

The genus Rickettsia, responsible for rickettsioses, includes strictly intracellular bacteria, subdivided into three subgroups: the spotted fever group (SFG), the typhus group (TG) and the scrub typhus group (STG). Most human rickettsioses are diagnosed on the basis of clinical evidence and epidemiological investigation. The manifestations of the main rickettsioses are summarised in Table 2.

3.1.2. Molecular diagnosis

As the clinical manifestations of rickettsioses are not specific, laboratory tests are necessary to confirm the diagnosis. The diagnosis relies mainly upon serology. The limit of this approach is the absence of antibodies in the early-phase disease and the existence of cross-reactions between the different Rickettsia spp. Molecular methods based on PCR have enabled the development of sensitive, specific and rapid tools for both detection and identification of rickettsiae from various samples: blood sample, biopsy specimen of the eschar, and arthropods (ticks, flea, lice). Prior to test, blood must be held at ambient temperature until cells are sedimented and rickettsiae are sought in the leucocyte cell buffy coat. PCR assays can be very useful because infection can be detected before seroconversion or positive culture has occurred.

For rickettsioses, detection strategies based on recognition of sequences within the genes encoding the 16S rRNA gene, a 17-kDa protein, the citrate synthase, the outer membrane proteins rOmpA and rOmpB, the surface cell antigen 4 and the surface cell antigen 1 have been developed [3,5]. The complete sequences of R. conorii, R. rickettsii, R. prowazekii, R. typhi, R. felis, R. africae, R. slovaca, Rickettsia akari, Rickettsia massiliae, Rickettsia bellii, Rickettsia canadensis and Rickettsia sibirica genomes have provided an important source of gene sequences for PCR-based assays [6–10].
Table 2  
Clinical symptoms, aetiological agent, arthropod-vector and distribution of currently recognised rickettsiosis

<table>
<thead>
<tr>
<th>Disease</th>
<th>Aetiological agent</th>
<th>Arthropod associated</th>
<th>Distribution</th>
<th>Presence of rash (%) (positive)</th>
<th>Eschar (% positive)</th>
<th>Local nodes</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spotted fever group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMSF</td>
<td><em>R. rickettsii</em></td>
<td>Dermacentor spp.</td>
<td>America</td>
<td>90</td>
<td>Very rare</td>
<td>No</td>
<td>1–5</td>
</tr>
<tr>
<td>MSF</td>
<td><em>R. conorii conorii</em></td>
<td>Rhicophilus sanguineus</td>
<td>Mediterranean countries</td>
<td>97</td>
<td>Rare</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>ATBF</td>
<td><em>R. africæ</em></td>
<td>Amblyoma spp.</td>
<td>Africa</td>
<td>30</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Yes, scalp</td>
<td>Low</td>
</tr>
<tr>
<td>TIBOLA DEBONEL</td>
<td><em>R. slovaca</em></td>
<td>Dermacentor marginatus</td>
<td>France, Portugal</td>
<td>?</td>
<td>Yes, cervical</td>
<td>Yes, No</td>
<td></td>
</tr>
<tr>
<td>LAR</td>
<td><em>R. sibirica</em></td>
<td>Hylomma asiaticum</td>
<td>Mongolia, France</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Astrakhan spotted fever</td>
<td><em>R. conorii</em></td>
<td>Rhipicaphalus pumilio</td>
<td>Astrakhan</td>
<td>100</td>
<td>23</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Israeli spotted fever</td>
<td><em>R. conorii</em></td>
<td>Rhipicaphalus sanguineus</td>
<td>Israel</td>
<td>100</td>
<td>No</td>
<td>No</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Rickettsialpox</td>
<td><em>R. akari</em></td>
<td>Allodermansus spp.</td>
<td>United States, Ukraine, Slovenia</td>
<td>100</td>
<td>83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Yes</td>
<td>Low</td>
</tr>
<tr>
<td>Queensland tick typhus</td>
<td><em>R. australis</em></td>
<td>Ixodes holoclycus</td>
<td>Australia</td>
<td>100</td>
<td>65</td>
<td>Yes</td>
<td>Low</td>
</tr>
<tr>
<td>Flinders Island spotted fever</td>
<td><em>R. honei</em></td>
<td>?</td>
<td>Tasmania</td>
<td>85</td>
<td>28</td>
<td>Yes</td>
<td>Low</td>
</tr>
<tr>
<td>Japanese spotted fever</td>
<td><em>R. japonica</em></td>
<td>Haemophylalis spp.</td>
<td>Japan</td>
<td>100</td>
<td>48</td>
<td>No</td>
<td>Low</td>
</tr>
<tr>
<td>Siberian tick typhus</td>
<td><em>R. sibirica</em></td>
<td>Dermacentor spp.</td>
<td>Siberia, Russia, China</td>
<td>100</td>
<td>77</td>
<td>Yes</td>
<td>Low</td>
</tr>
<tr>
<td>Unnamed spotted fever</td>
<td><em>R. helvetica</em></td>
<td>Ixodes ricinus</td>
<td>Switzerland, France, China</td>
<td>?</td>
<td>?</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Unnamed spotted fever</td>
<td><em>R. aeshchlimannii</em></td>
<td>Hyloma marginatus</td>
<td>France, Morocco, South Africa</td>
<td>Yes</td>
<td>Yes</td>
<td>?</td>
<td>No</td>
</tr>
<tr>
<td>Unnamed spotted fever</td>
<td><em>R. parkeri</em></td>
<td>Amblyoma spp.</td>
<td>United States, France, Spain, Italy, Portugal</td>
<td>Yes</td>
<td>Yes</td>
<td>?</td>
<td>Low</td>
</tr>
<tr>
<td>Unnamed spotted fever</td>
<td><em>R. massilae</em></td>
<td><em>R. sanguineus</em></td>
<td>?</td>
<td>Yes</td>
<td>Yes</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Unnamed spotted fever</td>
<td><em>R. helongjiangensis</em></td>
<td>Ctenocephalides felis and <em>C. canis</em></td>
<td>China</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>?</td>
</tr>
<tr>
<td>Unnamed spotted fever</td>
<td><em>R. felis</em></td>
<td>Dermacentor spp.</td>
<td>France, Thailand, Germany, Spain, Brazil, Texas, Mexico</td>
<td>Yes</td>
<td>Yes</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Typhus group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epidemic typhus</td>
<td><em>R. prowazekii</em></td>
<td>Pediculus humanus corporis</td>
<td>Worldwide</td>
<td>40</td>
<td>No</td>
<td>No</td>
<td>2–30</td>
</tr>
<tr>
<td>Marine typhus</td>
<td><em>R. typhi</em></td>
<td>Xenopsylla cheopis</td>
<td>Worldwide</td>
<td>50</td>
<td>No</td>
<td>No</td>
<td>Low</td>
</tr>
<tr>
<td>Scrub typhus group</td>
<td><em>O. tsutsugarmushi</em></td>
<td>Leptotrombidium deline</td>
<td>Asia, Pacific</td>
<td>50</td>
<td>Yes</td>
<td>Yes</td>
<td>2-5</td>
</tr>
</tbody>
</table>

RMSF, Rocky Mountain spotted fever; MSF, Mediterranean spotted fever; ATBF, African tick bite fever; TIBOLA, tickborne lymphadenitis; DEBONEL, Dermacentor-borne necrosis-erythema-lymphadenopathy; LAR, lymphangitis-associated rickettsiosis disease.

<sup>a</sup> Multiple eschar.

Several PCR techniques have been used for the diagnosis of rickettsioses. Nested-PCR techniques have been described in order to increase the analytical sensitivity of the test but the risk of DNA-amplicon contamination could not be ruled out using this approach [11]. Recently, quantitative real-time PCR assays have been developed for the diagnosis of infections caused by *Orientalia tsutsugamushi*, SFG and TG rickettsiae [12–14]. In addition, quantitative real-time PCR assays using species-specific probes targeting *Rickettsia prowazekii*, *Rickettsia typhi* and *Rickettsia felis* are also available [15,16].

A PCR assay with increased sensitivity, named ‘suicide-PCR’, has been reported [17–21]. This test was mainly designed to detect DNA from samples when regular PCR has a poor sensitivity. This technique is a nested PCR using a single-use primer targeting a gene never amplified previously in the laboratory. Such a procedure avoids ‘vertical’ contamination by amplicons from previous assays. All positive PCR products are sequenced to identify the causative agent. Suicide-PCR has been successful with EDTA-blood, serum, skin, lymph node specimens and palaeomicrobiology.
Table 3
Bartonella spp. and related systemic infections in humans

<table>
<thead>
<tr>
<th>Disease</th>
<th>Clinical presentation</th>
<th>Bartonella spp. involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic bacteraemia</td>
<td>Fever, headache, leg pain, thrombocytopenia</td>
<td>B. quintana</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>Blood culture negative endocarditis</td>
<td>B. quintana, B. henselae, B. elizabethae, B. vinsonii arapensis, B. vinsonii berkhoftii, B. kohlerae, B. alsatica</td>
</tr>
<tr>
<td>Trench fever</td>
<td>Relapsing fever</td>
<td>B. quintana</td>
</tr>
<tr>
<td>Oroya fever</td>
<td>Acute febrile haemolytic anaemia</td>
<td>B. bacilliformis</td>
</tr>
<tr>
<td>Bacillary angiomatosis</td>
<td>Red and papular cutaneous lesion</td>
<td>B. quintana, B. henselae</td>
</tr>
<tr>
<td>Bacillary peliosis</td>
<td>Abdominal pain, fever, hepatosplenomegaly</td>
<td>B. henselae</td>
</tr>
<tr>
<td>Visceral cat scratch disease</td>
<td>Lympadenopathy, hepatosplenomegaly</td>
<td>B. henselae</td>
</tr>
</tbody>
</table>

3.2. Q fever

3.2.1. Clinical context

Q fever is a potentially severe and worldwide zoonosis due to Coxiella burnetii [22] that can occur in large outbreaks of acute infections. The common sources of infection are cattle, goats and sheep. Humans are most often infected by inhalation of contaminated aerosols [22]. The disease is commonly divided into acute and chronic infections [22]. In symptomatic acute Q fever, the most common clinical manifestations are fever, pneumonia and hepatitis. Chronic Q fever develops in predisposed patients [22]. The main clinical presentation of the chronic form is represented by BCNE, which occurs in patients with previous valvular damage after an episode of acute Q fever. Less frequently, cases of vascular infections in patients with aneurysm or a vascular prosthesis are found. Acute or chronic forms of Q fever have been reported in pregnant women. Most of the cases are asymptomatic but complications can present, such as in utero foetal death or hypotrophia [22].

3.2.2. Molecular diagnosis

The diagnosis of Q fever relies mainly on serological examination. In the absence of a specific diagnosis based on serology, chronic Q fever is fatal. However, diagnosis is often delayed because the test is not widely available. The LightCycler Nested PCR (LCN-PCR), a rapid nested-PCR assay that uses serum as a specimen and the LightCycler as a thermal cycler, targeting a multicopy 20-copy htpAB-associated element sequence has been adapted for the diagnosis of both acute and chronic Q fever [23,24]. Indeed, serum sampled early during the disease has been shown to be useful for the diagnosis of acute Q fever in the first 2 weeks of the disease but should be reserved for seronegative patients in the next 2 weeks and not used later than 4 weeks following onset, when serology is highly sensitive [23]. The LCN-PCR assay may be helpful in establishing an early diagnosis of chronic Q fever [24]. Due to its high sensitivity and specificity, the repetitive element, IS 11-11, is the best target gene for the detection of C. burnetti in patients with active Q fever [3]. Presently, the genome of C. burnetti is available, allowing a large choice of DNA targets [25].

3.3. Bartonelloses

3.3.1. Clinical context

Bartonella spp. have been recognised as further causative agents of human bloodstream infections, which are summarised in Table 3.

3.3.2. Molecular diagnosis

Several targets have been successfully used to detect Bartonella spp. from clinical samples such as the citrate synthase gene, the 16S–23S rRNA intergenic spacer recognition, the ribosynthase H9251 chain gene, the heat shock protein, the RNA polymerase beta-subunit-encoding gene, the gene encoding the PAP31 and 35-kDa proteins, and the cell division protein gene [3]. As the gene encoding the PAP31 is present in multicopy, the PCR targeting this gene is more sensitive [3].

Real-time nested-PCR assay performed on a LightCycler apparatus (LCN-PCR) is a valuable tool that can shorten the delay in the diagnosis of Bartonella endocarditis [26]. Moreover, it has been suggested that this technique might be useful for other systemic Bartonella infections. The complete sequences of Bartonella henselae and Bartonella quintana genomes have provided an important source of gene sequences for PCR-based assays [27].

3.4. Whipple’s disease

3.4.1. Clinical context

The clinical picture of Whipple’s disease (WD) is polymorphic and non-specific [28]. In the past, WD was considered mainly as a digestive disease. Roughly 15% of cases lack classic signs and symptoms. Some patients have been diagnosed in the absence of classic signs when typical histological lesions were found in PAS stains of small-bowel biopsies. Thus, there are several presentations linked
to *T. whipplei* infection: classic WD marked by histological lesions in the gastrointestinal tract in association with diverse clinical manifestations; blood-culture-negative endocarditis; and isolated neurological infection due to *T. whipplei*.

### 3.4.2. Molecular diagnosis

One of the limits of the histological analysis of biopsies using the periodic acid-Schiff is the lack of specificity. PCR may be used to detect *T. whipplei* in samples from a variety of tissue types and body fluids, blood samples, saliva and stools [28]. The DNA extraction is a critical step. Various protocols have been proposed [28]. Initially, PCR assays targeting the 16S rRNA gene and 16S–23S intergenic regions of the *T. whipplei* gene were used [28]. Based on genome analysis, quantitative real-time PCR assay targeting repeated sequences of *T. whipplei* has substantially enhanced the sensitivity of the assay without altering its specificity [4]. Discrepancies between laboratories suggest that PCR results obtained with ‘home-made PCR’ must be interpreted with caution. The high frequencies of positive PCR tests from people without WD have primarily been obtained using nested or semi-nested techniques, which carry a high risk of contamination [11,28]. However, it is important to pay attention to a positive PCR assay, as suggested by the death of a patient in whom one of three PCR tests was positive on a joint fluid sample but whose duodenal biopsies were negative on PAS-staining [29]. The diagnosis of WD was excluded for this patient, yet the autopsy revealed WD.

### 4. Infective endocarditis

#### 4.1. Clinical context

The diagnosis of infective endocarditis (IE) is most often based upon the detection of vegetation on the cardiac valves using echocardiography and positive blood culture [30]. However, in numerous situations, blood culture and/or echocardiography are not able to confirm the diagnosis. To help physicians in establishing the final diagnosis of IE, diagnostic scores have been defined. The Duke score comprises the main criteria presently used and includes major criteria (i.e., blood culture positive for IE, echocardiographic findings consistent with IE, valvular regurgitation, and *C. burnetii* antiphase I IgG titers of ≥ 800) and minor criteria (i.e., predisposition, fever, vascular and immunological phenomena, and growth of organism in blood cultures but failure to meet a major criterion) [31].

Culture of blood is critical for the diagnosis of IE. It is now clearly established that three samples containing 40 mL of blood, obtained within a time of 4 h is enough to detect usual micro-organisms [32]. Thus, extensive blood culture and subculturing is not necessary, and HACEK group organisms are recovered by regular 5 days’ incubation. Specialised culture methods are useful when regular blood cultures fail to recover the aetiological agents such as the shell-vial cell-culture assay. This allows recovery of *C. burnetii, Bartonella* spp., *T. whipplei* and *Brucella* spp.

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![Fig. 2. Strategy for the interpretation of PCR results performed on cardiac valves.](image)

(* Repetition of PCR targeting the 16S rRNA sequence in case of high suspicion of endocarditis.)
However, this technique is restricted to specialised laboratories.

IE remains a diagnostic challenge as aetiological diagnosis is critical to select a proper treatment, and because the fatality rate remains high (20–25%) [34]. The proportion of IE without aetiological diagnosis (4.5–48%) varies from country to country and from different centres in the same country [34]. BCNE is defined as an IE without aetiology after three blood samples inoculated on standard medium. Schematically, half of BCNE is due to previous antibiotic therapy; the other half is due to intracellular bacteria or fastidious growing bacteria. Infection of the right heart, specifically on pacemakers, is also responsible for BCNE.

4.2. Molecular diagnosis

The diagnostic strategy for BCNE starts by serological testing of fastidious agents. As the minimum, *Coxiella burnetii* and *Bartonella* spp. should be tested. A single serum is sufficient, as endocarditis is a chronic disease associated with high-level IgG antibodies. When serology is not performed or is negative, molecular diagnosis should be undertaken.

4.2.1. Blood specimen

For the universal detection of bacteria in blood, broad-range PCR assays based on 16S rRNA amplification and sequencing have been described. Although the sensitivity and specificity of this approach is established in resected valves (see below), the usefulness of this technique in blood is still debatable [30].

Recently, a commercialised kit from Roche (Mannheim, Germany) has been proposed for both rapid genomic DNA preparation and multiplex PCR amplification from blood. Genomic DNA is directly released from blood cells in a single step by adding a specific reagent directly to blood samples. The genomic DNA can then be used immediately in PCR amplification of multiple gene targets.

Real-time PCR, performed on sera, has been successfully developed for the diagnosis of *Bartonella* IE [26] and Q fever endocarditis [24].

4.2.2. Cardiac valves

Molecular techniques using broad-range PCR targeting the 16S rRNA performed on resected valves with subsequent sequencing of the amplicon and gene analysis with comparison to the data bank have allowed new aetiological diagnoses of BCNE [35]. The bacteria detected are mainly streptococci in patients with previous antibiotic treatment, and fastidious bacteria including *Granulicatella* spp., *Abiotrophia* spp., *C. burnetii*, *T. whipplei*, *Mycoplasma hominis* and *Mycobacterium* spp. [30,35–41]. It is very important to emphasise that DNA from the causative agent can persist months to years after clinical cure [42,43]. Thus, the link between the current IE episode and the amplified DNA needs to be carefully checked.

### Table 4

<table>
<thead>
<tr>
<th>Genes, probes and specific primers used for amplification of 10 bacterial bioterrorism agents</th>
<th>Bacteria</th>
<th>Target gene</th>
<th>Primer name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus anthracis</td>
<td>pag</td>
<td>pag</td>
<td>pag</td>
<td>AGGCTCGAACTGGAGTAGCA</td>
<td>CCGCCTTTCTACCAGATTT</td>
<td>TACCGCAAATTCAAGAAACAACTGC</td>
</tr>
<tr>
<td>Yersinia pestis</td>
<td>pla</td>
<td>pla</td>
<td>pla</td>
<td>ATGGAGCTTATACCGGAAAC</td>
<td>GCGATACTGGCCTGCAAG</td>
<td>AAGGAGTGCGGGTAATAGGTTATAA</td>
</tr>
<tr>
<td>Francisella tularensis</td>
<td>fopA</td>
<td>fopA</td>
<td>fopA</td>
<td>TAATAATTTCATTGCTCCTTTTG</td>
<td>TTCTATCTTGAGGACCCCAA</td>
<td>TTGCAAATACTTATAGCGCTTTGACT</td>
</tr>
<tr>
<td>Brucella spp.</td>
<td>omp19</td>
<td>omp19</td>
<td>omp19</td>
<td>CCGGTGAACTGGCTAATCT</td>
<td>TGAAGAATAGAGCGAGGCAA</td>
<td>AATGGCAAGCAACTCGTCCTTTAC</td>
</tr>
<tr>
<td>Burkholderia mallei</td>
<td>fliC</td>
<td>fliC</td>
<td>fliC</td>
<td>GACGCTGGCGCTGTCGA</td>
<td>CGGCTTGTTGACCGCGTT'</td>
<td>AACCAGCGCGCTGTCCGCGAC</td>
</tr>
</tbody>
</table>

It is very important to emphasise that DNA from the causative agent can persist months to years after clinical cure [42,43]. Thus, the link between the current IE episode and the amplified DNA needs to be carefully checked.
However, based on our experience, we suggest that nucleic acid amplification-based detection and identification of IE micro-organisms from cardiac valves should be included in the Duke criteria (i.e., a micro-organism can be demonstrated by culture, histologic examination or molecular-based techniques to fulfil a pathologic criterion and thus to contribute to the definite diagnosis of IE) [34]. This specificity may be raised to 100% if interpreted following a predefined procedure (Fig. 2) [38].

5. Bioterrorism attack

Rapid and accurate assays for microbial identification are essential to ensure proper medical intervention in the case of suspected release of bioterrorism agents. The bacterial agents are listed as category A (Bacillus anthracis, Francisella tularensis and Yersinia pestis) and category B (Brucella spp., Burkholderia mallei, Burkholderia pseudomallei, Chlamydia psittaci, C. burnetii, Rickettsia prowazekii, and Rickettsia rickettsii) by the Centers for Disease Control and Prevention (CDC). Many of these agents are fastidious micro-organisms. Culture of these bacteria requires a L3 biosafety laboratory but the success of isolation is not guaranteed. Molecular diagnosis is well adapted to this situation. However, there are several limitations to this approach. The limited circulation of many of these micro-organisms precludes the ready availability of positive controls. Several micro-organisms would have to be tested simultaneously. False-positive results due to contamination from positive controls should be avoided.

The first rapid molecular tests were limited to the detection of B. anthracis, Brucella spp., F. tularensis and Y. pestis [44]. Recently, a quantitative molecular tool targeting the ten bioterrorism-related bacteria was developed using specific PCR and serial dilutions of a plasmid suspension in molecular assays. Designed primers and probes allowed molecular detection, in either single or multiplex PCR assays of agent-specific targets and in both conventional and real-time PCR assays (Table 4). The plasmids, which contain specific sequences from the agents, are used as positive controls. An ‘exogenic sequence’, introducing a NotI restriction site was incorporated in the native sequences of the bioterrorism agents inserted in plasmids. Thus, false-positive results due to contamination by the positive control could be easily detected by sequencing and eliminated by digestion with NotI [44].

6. Conclusions

PCR is a great step forward in the diagnosis of infectious diseases. Technology is rapidly evolving and the genomes provide sequences for each specific question. A major limitation of molecular techniques used for detection and identification of bacteria is the lack of the simultaneous provision of the antimicrobial susceptibility pattern. In addition, it is necessary to be cautious, and technical procedures must be strictly applied and controls systematically used.

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References


