Myocarditis is an inflammatory disease of the myocardium associated with cardiac dysfunction. The natural history of myocarditis is frequently characterised by the evolution in dilated cardiomyopathy. Due to its variable clinical manifestation from latent to very severe clinical forms, such as acute congestive heart failure and sudden death, its prevalence is still unknown and probably underestimated. In spite of the development of various diagnostic modalities, early and definite diagnosis of myocarditis still depends on the detection of inflammatory infiltrates in endomyocardial biopsy specimens according to the Dallas criteria. Routine application of immunohistochemistry, used for identification and characterisation of inflammatory cell populations, has now significantly increased the sensitivity of the diagnosis of inflammatory cardiomyopathy. Various molecular techniques, such as PCR, gene sequencing and real-time PCR, often applied on the same endomyocardial specimen, have become an essential part of the diagnostic armamentarium for rapid, specific and sensitive identification of infective agents. The correct application of molecular techniques will allow increasingly more information to be obtained: new epidemiology, new patient risk stratification and overall more appropriate medical treatment.

1. Introduction: role of endomyocardial biopsy

According to the current WHO classification of cardiomyopathies, myocarditis is an inflammatory heart muscle disease associated with cardiac dysfunction, and dilated cardiomyopathy (DC) may represent the chronic phase of the disease [1]. Clinical features of the disease are variable including unexplained congestive heart failure, chest pain mimicking myocardial infarction [2], arrhythmias, syncope and sudden death. Sudden death may occur both in the active or healed phases as a consequence of life-threatening ventricular arrhythmias that develop mostly in the setting of an unstable vulnerable myocardial substrate, namely inflammatory infiltrates, interstitial edema, myocardial necrosis and fibrosis. Patchy inflammatory infiltrate, in the form of a starry sky-like feature (<14 leucocytes/mm²), and not necessarily associated with myocyte necrosis, is a frequent observation. This subtle substrate, together with the possible inflammatory involvement of the conduction system, seems highly arrhythmogenic and may account for unexpected arrhythmic cardiac arrest [3].

Heterogeneity of symptoms, including subclinical or asymptomatic forms, could be the reason why the prevalence of myocarditis is still unknown and probably underestimated. The large spectrum of clinical forms—from subclinical to severe—depends on several factors such as genetic determinants of infective agents, the genetics, age and gender of the host, and host immunocompetence.

In spite of the development of various diagnostic modalities, early and definite diagnosis of myocarditis still depends on the detection of inflammatory infiltrates in endomyocardial biopsy (EMB) specimens according to the Dallas criteria [4]. The major limitation of EMB remains the low sensitivity due to sampling error, particularly in the presence of focal disease. The size and number of the heart biopsy specimens as well as the processing of
myocarditis of ~50% using four to five biopsy samples. When 17 biopsy specimens per case were considered, the sensitivity reached 79% [7]. It has also been shown that serial sectioning and multiple level examination of EMBs increase the sensitivity of EMB in the evaluation of myocarditis [9, 10], thus facilitating the diagnosis of focal myocarditis with a rate of sensitivity similar to that in diffuse myocarditis [10].

Although some authors have demonstrated that biventricular EMB (obtaining several samples from multiple areas of the left and right ventricles) may improve the sensitivity in the detection of myocarditis [11], it should be emphasised that this aggressive approach increases the risk of complications. However most experts in the field agree that an actual increase in the sensitivity of EMB has now been reached using immunohistochemistry together with routine histology. A large panel of monoclonal and polyclonal antibodies is now mandatory to identify and characterize the inflammatory cell population as well as the activated immunological processes [12–15].

Even though sampling and processing techniques have significantly increased the sensitivity of EMB in the evaluation of myocarditis, morphological analysis today is still greatly limited in the detection of infective pathogens, particularly viral agents, the commonest cause of inflammatory cardiomyopathy (IC). Viral myocarditides usually lack specific cytopathic effects (Fig. 1a,b), especially those forms of cytomegaloviral myocarditis, these effects, when observed, neither necessarily imply the presence nor are useful in detecting the type of virus, since they may represent degenerative changes or myocyte nuclear hyperplasia.

2. Etiology

IC has multiple causes, both non-infectious and infectious, and viruses account for the disease in the majority of paediatric cases (Table 1). Different cardiotropic viruses have been implicated in more than 50% of paediatric cases with IC [16, 17]. The

Table 1
Myocarditis: infective agents

<table>
<thead>
<tr>
<th>Viruses RNA</th>
<th>Picornavirus (entero, rhino); orthomyxovirus (influenza A,B); paramyxovirus (rubeola, mumps); retrovirus (HIV-1); hepatitis C virus (HCV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viruses DNA</td>
<td>Adenovirus, herpesvirus (cytomegal, Epstein–Barr virus, varicella-zoster), Parvovirus Big</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Staphylococcus, Streptococcus, Pneumococcus, Meningococcus, Gonococcus, Salmonella, Corynebacterium diphtheriae, Haemophilus influenzae, Mycoplasma pneumoniae, Bracella species</td>
</tr>
<tr>
<td>Mycobacteria</td>
<td><em>Tuberculosis</em>, <em>Avium intracellulare</em>, <em>Leprae</em></td>
</tr>
<tr>
<td>Fungi</td>
<td>Aspergillus, Candida, Actinomyces, Blastomyces, Cryptococcus, Histoplasma, Parisedia gondii, Trypanosoma cruzi</td>
</tr>
<tr>
<td>Protozoa</td>
<td>Coxiella burnetti (Q fever), <em>Rickettsia rickettsii</em>, <em>Rickettsia tsutsugamushi</em></td>
</tr>
<tr>
<td>Rickettsiae</td>
<td><em>Trachomatis</em>, <em>Psittaci</em></td>
</tr>
<tr>
<td>Chlamydiae</td>
<td><em>Trichinella spiralis</em></td>
</tr>
</tbody>
</table>
remaining forms may be triggered by other environmental factors such as toxins, alcohol, cytotoxic chemotherapy, metabolic abnormalities or more frequently sustained by immunologic mechanisms. These latter may characterize the autoimmune myocarditis ab initio or more often are the consequence of viral myocarditis itself [18].

Among cardiotropic viral agents, coxsackie viruses are thought to be most frequently implicated [19,20]. Group B coxsackie viruses consist of six serotypes within the enterovirus genus *Picornaviridae*, a large family of single-stranded positive-sense RNA viruses. In the WHO record during the 10-year period from 1975 through 1985, the coxsackie B viruses represent the most frequent inflammatory agents in cardiovascular disease (34.6 per 1000), followed by influenza B virus (17.4 per 1000), influenza A (11.7 per 1000), coxsackie A (9.1 per 1000) and cytomegalovirus (CMV) (8.0 per 1000) [21].

The prevalence of the enteroviruses as infective agents associated with myocarditis has been reported in several clinical studies [19–22]. Patients with IC had significantly higher serological titres when compared with controls [23–25]. In contrast, a coxsackie virus infection could be proven in only 2% of consecutively studied myocarditis patients in a 5-year study at a military hospital in Finland, where traditional enteroviral diagnostic methods were used [26]. Variable aetiology was found and was mainly represented by vaccine inoculation, followed by other infective agents such as adenovirus, Epstein–Barr virus, *Myoplasma pneumoniae* and *Chlamydia*.

Using molecular techniques, the frequency of myocardial enteroviral infection is now estimated in ~30–50% of cases with infectious etiology [16,17,27,28]. Various respiratory tract viruses—adenoviruses, Epstein–Barr viruses (EBV), influenza viruses, etc.—may cause myocarditis at variable frequencies [20,21,26,29]. In particular, adenoviruses have been shown to be an important cause of myocarditis and dilated cardiomyopathy both in childhood [16,17] and adulthood [30].

It is noteworthy that rhinovirus-associated myocarditis has been rarely reported in the literature [31], although several cases have been registered with the U.S. Centers for Disease Control [21].

Cytomegalovirus (CMV) is a recognized cause of acute infectious myocarditis in the herpes group of viruses, although CMV is considered to be uncommon in previously healthy people [32]. However some authors detected a CMV-specific genome in the myocytes of EMBs in up to 15% of patients with acute myopericarditis [33]. Therefore CMV infection may be considered a more frequent cause of myocarditis than was previously thought. CMV infection is a peculiar viral disease in transplant recipients, with multiorgan involvement [32–34], since specific cytopathic features are only exceptionally observed. In transplanted patients, treated with ganciclovir for a previous CMV infection, the biopsy features of CMV myocarditis appear modified since the infected cells often do not show the characteristic cytomegaly, and the intranuclear inclusions may not be basophilic [34–36].

During or following mumps, mild or subclinical myocardial infection may be present with 15% of cases showing ST-T wave changes. Mumps-induced myocarditis has been demonstrated to be the first step in the pathogenesis of endocardial fibroelastosis [17,37]. In recent years the incidence of the disease, which was previously considered a significant cause of infant mortality, has dramatically declined, probably due to mumps vaccine.

Parvovirus B19, the causative agent of erythema infec-
tiosum, also called fifth disease, has been reported to be a rare but severe cause of myocarditis in infants and children [38–40]. The virus has also been considered as being responsible for hydrops fetalis and foetal death [41,42]. The B19 receptor (erythrocyte P antigen) has been recognized on foetal myocardial cells [38,42] suggesting that intrauterine myocarditis contributes to the development of fetal hydrops after parvovirus B19 infection.

The importance of hepatitis C virus (HCV) in patients with myocarditis and DC has recently been stressed [43–45]. Myocyte tropism has also been demonstrated by Takeda et al. using in situ hybridisation [46] and recently by our group using immunohistochemistry with TORDJI-22 monoclonal antibody [45].

IC caused by other non-viral infective agents is nowadays rare. It usually occurs in immunosuppressed patients with secondary involvement of the myocardium.

Myocarditis is the most common cause of death in diphtheria by the action of exotoxin produced by *Corynebacterium diphtheriae*. Although still a cause of morbidity and mortality in developing countries [47], diphtheria is now rare in the West, most probably because of vaccination. It re-appeared in Scandinavia in the mid-1980s, mostly in alcoholics [48].

Non-rheumatic myocarditis may be a complication of beta-haemolytic streptococci (*Streptococcus pyogenes*), due to bacterial exotoxins [49]. Rheumatic carditis with involvement of both the valvular apparatus and perimyocardium accounts for the majority of pediatric hospitalizations and cardiac deaths worldwide, particularly in developing countries. Immune responses to group A streptococcal antigens during pharyngitis, resulting in antibodies and immunocompetent cells that cross-react with myocardial antigens, are thought to be central to the pathogenesis of rheumatic carditis. The recent finding of enteroviral RNA replication and protein synthesis in valvular tissue from patients with chronic rheumatic heart disease may provide intriguing evidence for a viral etiology or cofactor, at least in some cases [50]. Bacterial endotoxin is also considered to play a role in meningococcal myocarditis. Myocarditis has been found in up to 78% of cases of meningococcal septicaemia with marked endotoxaemia [51].

Even though rarely, myocarditis has been found in *Salmonella septicaemia* caused by *Salmonella typhi* or...
paratyphi. Yersiniosis myocarditis, probably more frequent than is commonly thought, is in most cases a quite mild form and considered to be immune in the pathogenesis [52]. Myocarditis is a well documented complication (1–8% of cases) in Borrelia burgdorferi infection. Involvement of the specialized myocardium may account the onset of atrioventricular block [53]. Mycoplasma pneumoniae infection is commonly accompanied by mild myocarditis with up to 33% of cases exhibiting ECG changes [54].

Chlamydia psittaci infection is complicated by subclinical or asymptomatic myocarditis in 5–15% of cases [55], but cardiac insufficiency may be marked [56]. In Chlamydia trachomatis infection myocarditis is rare. A few published cases have been reported in the literature, even fatal, with the majority of these involving small children [55]. Chlamydia pneumoniae infection combined with myocarditis has been also described as a mild form [57], although one case of sudden death in a young athlete [58].

Cardiac involvement is often found in the setting of rickettsial infection with vasculitis as a prominent feature, because the rickettsiae have special tropism for endothelial cells. Even though endocarditis is considered more common, myocarditis may also present in Q fever caused by Coxiella burnetii [59].

Trypanozona cruzi (Chagas’ disease) is a well-recognized cause of myocarditis and cardiomyopathy in both urban and rural areas of South America [60]. Also the African trypanosomes, Trypanozona gambiense and Trypanozona rhodesiense, occasionally cause myocarditis and inflammatory cardiomyopathy [61].

Toxoplasma gondii poses significant problems among recipients of cardiac transplants. A study reported that 57% of transplanted patients lacking antibodies to that agent developed toxoplasma myocarditis [62]. However, toxoplasmosis may become reactivated in antibody-positive transplant recipients, and myocarditis has been reported in 4–53% of transplant cases. This great variation rate is probably due to differences in antibody testing methods [63]. After the introduction of pyrimethamine prophylaxis, this complication has decreased substantially.

Myocarditis seems to be quite frequent in AIDS patients, with a rate of 45–52% of cases [64]. Cardiac dysfunction in AIDS may, however, have various forms of pathogenesis. The aetiology of myocarditis in AIDS is also variable and may be difficult to establish, especially in cases with multiple opportunistic infections. Viruses are considered to be the most common cause, such as the human immunodeficiency virus (HIV) itself and coxsackie viruses. In one autopsy study, Toxoplasma gondii infection of the myocardium was found in 12% of cases [65].

Fungal myocarditis frequently occurs in the setting of disseminated disease. The major fungal pathogen responsible for myocardial infection is Aspergillus fumigatus [66]. The incidence of invasive fungal disease has dramatically increased over the past few decades corresponding to the increasing number of immunocompromised patients. The major risk factors for severe fungal disease include administration of broad-spectrum antibiotics, corticosteroids and cytotoxic agents, invasive medical procedures and HIV. In this latter condition the association of fungal and other infective agents may be quite frequent [67]. Pre-mortem diagnosis of fungal myocarditis is difficult since clinical findings of myocardial involvement are often absent or ambiguous, blood cultures are often negative and other laboratory tests are not effective.

Among parasitic infections, Trichinella spiralis is most prone to cause myocarditis, however other elmins can infect the myocardium usually in the setting of multi-organ involvement (Fig. 2a–d).

3. Pathogenesis

Acute myocarditis, more frequently viral forms, can be resolved without sequela, however progression in the chronic form, DC, is not a rare event. Two different theories have been recognized to explain myocyte damage and the progression from acute myocarditis to chronic forms/DC: autoimmunity and direct cytotoxicity due to persistent viral infection.

3.1. Autoimmunity

In 1957 Witebsky et al., modelling on Koch’s postulates, proposed rationales to explain the autoimmune basis of clinical disease: an autoimmune response, as either humoral or cell-mediated, is recognized when a specific antigen is identified and when an analogous autoimmune response is induced in an experimental animal [68]. Aberrant cellular and humoral immune responses have been proposed to explain the progression of viral myocarditis.

3.1.1. Aberrant cellular immune response

The original discovery that T-cells play a major role in the pathogenesis of coxackie virus B infection was reported over two decades ago. Mice depleted of T-cells by pre-treatment with antithymocyte serum and then infected with coxackie virus B3 exhibit a marked reduction in myocardial disease. The degree of cardiac inflammation and necrosis was much less than that found in immunologically intact mice or in mice reconstituted with both bone marrow and thymus cells [69].

Inflammation of the heart muscle can be transferred into non-immunized recipient mice by purified T-cells from mice with active myocarditis [70].

3.1.2. Aberrant humoral immune response

The hypothesis that humoral immunity plays a role in the development of post-infectious myocarditis is derived from the finding that immunization with cardiac myosin in
The finding of cross-reactive epitopes between cardiac myosin and infectious agents confirms this concept [76–78]. Molecular mimicry has been proposed as an explanation for coxsackie virus B3-induced autoimmune myocarditis. The hypothesis is supported by data from studies of monoclonal antibodies directed against coxsackie virus group B that recognizes epitopes on murine cardiac myosin [79]. Comparison of the nucleotide sequence and the deduced amino acid sequence of viral precursor polyprotein with the sequence of murine cardiac myosin showed however a very low degree of genetic homology [80].

It is very likely that the development of autoimmune myocarditis following viral myocarditis is sustained by a complex interaction between cellular and humoral immune response as proposed by Rose and Hill [80].

Abnormal human leucocyte antigen (HLA) expression on endomyocardial biopsy [12,13,81] increased levels of circulating cytokines and cardiac autoantibodies [82,83] are similarly also found in human myocarditis/DC, highly suggestive of an immune pathogenic mechanisms of viral myocarditis in the clinical setting.

### 3.2. Persistent viral infection

Continuous virus replication or persistent viral infection in the heart could play a role in the maintenance and/or progression of disease.

Molecular investigations have shown coxsackie virus B3 to persist in mouse myocardium in different models for up to 56 days [84,85] and in hamsters for up to 180 days [85].
Viral RNA was found to persist from 80 days to 12 months in other non-productive picornavirus myocardium infections in mice [86]. Molecular studies in various experimental models show that enterovirus can persist in the chronic stages of myocarditis [87]. The mechanisms of enterovirus persistence so far are not well known. A defective virus, mainly resulting from the altered function of the viral RNA polymerase [88], is now considered to be one of the possible mechanisms of persisting enteroviral infection. Therefore acute myocytolytic inflammatory disease evolves to persistent infection with a defective virus, unable to induce cytolysis or elicit the host cellular immune response.

Tam and Messner recently demonstrated that viral RNA perseveres in a double-stranded form in a murine model of coxsackie virus B1 infection of skeletal muscle. It is possible that double-stranded RNA is resistant to ribonuclease activity and is connected to reduced viral antigen expression, which in turn minimizes immune-mediated killing of persistently infected cells [89]. Therefore, if coxsackie virus B3 persists in a double-stranded form, it may be responsible for the long half-life of viral RNA during the chronic phase of infection.

Recently some authors have demonstrated that coxsackie virus B3 protease 2A can directly cleave dystrophin in myocytes, these data support the idea of a mechanism of direct viral destruction during the pathogenesis of chronic myocarditis/DC [90].

The activation of mitogen-activated protein kinase (MAPK) as a consequence of the P21 guanosine triphosphatase-activating protein (RasGAP) cleavage, has been more recently suggested to also play a role in the development of chronic myocarditis. The MAPK pathway is a mechanism employed by different viruses for control of cell proliferation and survival [91]. During enteroviral replication, activation of MAPKs may participate in the mobilization of intracellular calcium and may thereby contribute to morphological and physiological destruction of infected myocytes [92].

Several studies have also documented viral persistence in the myocardium of patients with DC until end-stage disease requiring cardiac transplantation [28,93–95].

4. Diagnostic tools for infective myocarditis

Different diagnostic tools are known to detect infective agents responsible for IC. Non-viral infective agents are often morphologically distinctive and identifiable by direct microscopic examination with routine and special stains (Gram’s, Gomori’s, periodic acid-Schiff, Ziehl-Neelsen, etc.). For these forms microbiologic culture and other laboratory tests are considered important for definitive identification of causative infective agents.

For viruses, the main pathogens of IC, traditional diagnostic tools, including morphological analysis (see above), have various limits because of low sensitivity and specificity.

4.1. Culture

It is important to recognize that the traditional methods of pathogen identification by culture isolation, phenotypic expression via biochemical reactions and determination of antibiotic sensitivity remain the gold standard for ~90% of infectious disease pathogens. However many infective agents, particularly viruses, can not be cultivated as they are very difficult to isolate.

For many viruses, such as enteroviruses, isolation procedures are poorly standardized and virus isolation data may vary considerably among laboratories. The type and quality of the specimen, the timing of specimen collection, and storage before arrival in the laboratory can highly influence the sensitivity [96]. The choice of cell types used for virus isolation is also important. For example no single cell line supports the growth of all known enterovirus serotypes [97] and many serotypes of adenovirus do not replicate efficiently in conventional cell cultures (Hep-2 and A549 cells) [98].

Virus isolation from blood components is useful for providing evidence of systemic infection but not for providing information on specific organ disease.

Detection of enterovirus in the alimentary tract (throat swabs, rectal swabs, or stool specimens) allows only circumstantial evidence of etiology, since viral shedding at these sites may occur in asymptomatic subjects, especially in infants and during epidemic seasons [99].

Isolation of a virus, particularly of enterovirus from affected tissues of patients with severe inflammatory myocardial diseases, has seldom been successful [100–103].

4.2. Serology

Following the isolation of a virus, the identification and overall serotypic characterization can be determined by neutralization of infectivity with serotype-specific antisera. Serologic diagnosis requires demonstration of IgM antibody or a fourfold increase in IgG antibody titre when paired serologies are obtained and is therefore retrospective.

Every viral family includes, however, a large number of serotypes (among the family Picornaviridae, 66 serotypes have been identified for the genus enterovirus) and typing by neutralization, with reference antisera for all serotypes individually, is surely not practical. In order to overcome this problem for the enterovirus family, equine type-specific hyperimmune sera have been mixed to provide intersecting pools containing different combinations of individual antisera [96].

Serotypic identification of an enterovirus isolate generally takes 1–2 weeks and usually has little impact on patient
care. Consequently, in the clinical setting a generic diagnosis of 'enterovirus infection' it is often put forward without further specifying the serotype [97].

Another important limit of serological analysis is in the high background prevalence of these responses. This likely reflects the ubiquity of many viruses (enterovirus, adenovirus, etc.), which circulate seasonally among the general population. Moreover immunoglobulin M responses may frequently be detectable for 6 months or longer after acute infection (such as for enterovirus) [104], may decline very rapidly resulting in negative values after only a few weeks (such as parvovirus B19) [105] or can result as a false negative in immunodeficient persons or children younger than 4 years (such as for EBV) [106].

Direct detection of different antigens in peripheral blood leukocytes by immunocytochemical staining (antigenemia) is now used efficiently for the diagnosis of different infective agents, particularly for viruses. Direct detection of the lower matrix phosphoprotein (pp65) in peripheral blood leukocytes has now been demonstrated to be a very sensitive tool for the diagnosis and monitoring of CMV infection. However the tool is almost laborious and shows quite low sensitivity for detecting the very early or chronic phase of the disease [107].

4.3. Molecular biology

4.3.1. Etiological diagnosis of inflammatory cardiomyopathy

The development of molecular biological techniques, particularly amplification methods such as polymerase chain reaction (PCR) or nested-PCR, allows the detection of low copy of viral genomes even from an extremely small amount of tissue such as endomyocardial samples. PCR is an enzymatic amplification technique whereby very few copies of RNA or DNA sequences can be amplified more than a million-fold. This allows transformation of a target sequence (i.e. the pathogen in question) from very low numbers, as RNA viral genomes usually are, to literally millions of copies without cloning technology. The decision to develop and apply PCR for routine diagnosis of infective myocarditis must be considered in relation to the speed, sensitivity and specificity of more conventional culture and/or serological methods [16,17].

However it should be stressed that the presence of bacterial or viral nucleotide sequences does not automatically imply a direct role in the pathogenesis of myocarditis. Indeed an infective agent detected by molecular techniques, especially with PCR/nested-PCR, may not be a cofactor but just an innocent bystander. Therefore it is extremely important to use a molecular technique as a diagnostic tool ancillary to other mandatory investigations—clinical and morphological—and apply it with skilled expertise.

Enterovirus, particularly coxsackie virus B, was the first virus investigated in myocarditis/IC based on coxsackie virus B3 experimental studies that provided inferential data to implicate enteroviruses as etiologic agents in human myocarditis [20,108,109]. Several reports have investigated the presence of enteroviral genome using PCR, overcoming the limits of the non-specificity of slot-blot and the low sensitivity of in situ hybridization and technical problems particularly when RNA probes are used [110,111].

However enteroviral RNA amplification studies, performed either on EMB specimens or on fragments from whole heart, have produced controversial data [94,112–125].

In Table 2 the principal studies that investigated only the presence of enterovirus are reported: the rate of viral amplification ranged from 0 to 80% for myocarditis and from 0 to 57.9% for DC. The wide discrepancy in results could have different explanations. It may be due to the different detection procedures adopted by the investigators.

<table>
<thead>
<tr>
<th>Authors, year</th>
<th>Material</th>
<th>Myocarditis</th>
<th>DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weiss et al. [112], 1992</td>
<td>EMB</td>
<td>--</td>
<td>5/11 (45%)</td>
</tr>
<tr>
<td>Grasso et al. [113], 1992</td>
<td>NH</td>
<td>--</td>
<td>0/21 (0%)</td>
</tr>
<tr>
<td>Katsuragi et al. [114], 1993</td>
<td>NH</td>
<td>0/1 (0%)</td>
<td>3/11 (27%)</td>
</tr>
<tr>
<td>Schwaiger et al. [115], 1993</td>
<td>EMB</td>
<td>--</td>
<td>6/19 (31.6%)</td>
</tr>
<tr>
<td>Giacca et al. [116], 1994</td>
<td>EMB</td>
<td>1/3 (33%)</td>
<td>4/53 (7%)</td>
</tr>
<tr>
<td>Khan et al. [117], 1994</td>
<td>EMB</td>
<td>--</td>
<td>16/8 (50%)</td>
</tr>
<tr>
<td>Nicholson et al. [118], 1995</td>
<td>NH</td>
<td>5/6 (83%)</td>
<td>--</td>
</tr>
<tr>
<td>Ueno et al. [119], 1995</td>
<td>EMB</td>
<td>4/5 (80%)</td>
<td>7/42 (17%)</td>
</tr>
<tr>
<td>Andreoletti et al. [120], 1996</td>
<td>EMB</td>
<td>--</td>
<td>11/19 (57.9%)</td>
</tr>
<tr>
<td>Muir et al. [121], 1996</td>
<td>NH</td>
<td>6/31 (19%)</td>
<td>5/28 (18%)</td>
</tr>
<tr>
<td>Fujikawa et al. [122], 1996</td>
<td>EMB</td>
<td>--</td>
<td>9/21 (42.9%)</td>
</tr>
<tr>
<td>Archard et al. [123], 1998</td>
<td>EMB</td>
<td>18/45 (40%)</td>
<td>--</td>
</tr>
<tr>
<td>Pauschinger et al. [30], 1999</td>
<td>EMB</td>
<td>1/2 (50%)</td>
<td>1/27 (3%)</td>
</tr>
<tr>
<td>Calabrese et al. [94], 1999</td>
<td>NH</td>
<td>6/11 (54.5%)</td>
<td>3/8 (37.5%)</td>
</tr>
<tr>
<td>Li et al. [124], 2000</td>
<td>EMB</td>
<td>--</td>
<td>0/68 (0%)</td>
</tr>
</tbody>
</table>

DC, dilated cardiomyopathy; EMB, endomyocardial biopsy; F, female; M, male; NH, native heart.
as well as to the prevalence of enteroviruses in local populations and the stage of the disease. This discrepancy emphasizes the need for a standardization of protocols (RNA extraction, reverse transcriptase and PCR reaction) to achieve the same level of sensitivity as the PCR technique used in different molecular laboratories. More recently other cardiotropic viruses have been investigated using molecular techniques and it has been shown that enterovirus is not the sole viral cause of IC. Adenovirus, especially in pediatric myocarditis, is another key etiological agent that should be investigated when the etiology is sought: adenovirus were found to be more prevalent than enterovirus in the case series of Martin et al. [16] and as common as enterovirus in our recent study [17] performed on children where myocarditis was the most frequent cause of heart failure.

EMB may be technically difficult to carry out in children for a diagnosis of myocarditis, which frequently complicates infectious respiratory disorders.

Recently Towbin’s group have demonstrated that tracheal aspirates may be a useful substrate for identification of causative agents by PCR analysis in young patients with myocarditis and presumed pneumonitis. In this study all PCR performed on EMB specimens demonstrated results identical to those obtained by tracheal aspirate PCR [126].

Even though enterovirus and adenovirus are the principal infective agents detected in myocardial specimens from patients with IC, other cardiotropic viruses are now seen as important pathogens [43,127,128]. Thus a specimen from patients with clinical suspicion of IC should always be investigated for at least for eight cardiotropic viruses: enterovirus, adenovirus, CMV, EBV, parvovirus b19, HCV and influenza virus A and B. An exact etiological diagnosis, providing new prognostic information as well as specific therapeutic indications, is now extremely important for appropriate management of patients.

Viral infection represents an independent unfavourable prognostic factor in patients [27], particularly in the paediatric population, affected by IC either as acute myocarditis or DC [16,17]. In our experience all patients with viral PCR proven DC underwent heart transplantation or died within 1 year of diagnosis [17].

The importance of specific viral treatment in cases with proven molecular diagnosis has been recently stressed by several authors [17,28,82,129].

Apart from the ongoing European Study of Epidemiology and Treatment of Cardiac Inflammatory Disease (ESETCID) trial [130], the Myocarditis Treatment Trial is the only large multicenter randomized study to have ever been accomplished [131]. Patients in this trial, classified with active myocarditis based on the Dallas criteria, received immunosuppressive therapy (either azathioprine and prednisone or cyclosporine and prednisone vs. placebo) in addition to conventional therapy. The results were inconclusive without any hemodynamic or prognostic benefit after 6 months of immunosuppressive treatment. Patients were not, however, evaluated for the presence of microorganisms, and thus many of the patients treated with immunosuppressive therapy may have harboured viral material, not detected by routine morphology. It is well known that, when there is active viral replication, immunosuppression may be harmful. Our retrospective study confirms the inefficacy of immunosuppressive therapy in patients with per proven viral myocarditis [83]. Immunosuppressive therapy has been demonstrated to have beneficial effects only when the autoimmune reaction is associated with IC [85,132].

Different molecular strategies have now been developed and applied to improve identification of an infective agent thus achieving a more complete etiological diagnosis.

Positive PCR results obtained on EMB should be always investigated on a blood sample collected at the time of biopsy. A type of algorithm might be developed for conclusive molecular diagnosis of myocarditis. The absence of a viral genome in the blood sample rules out the possibility of passive blood contamination while viral blood positivity requires additional investigation using quantitative PCR analysis. The presence of viral genomic copies, significantly less in the EMB than in blood, does not necessarily mean that the heart is infected due to viremia, a situation that has recently been reported in a case of fatal parvovirus myocarditis [40].

On more than 600 EMBs (from 1992 to 2001) from patients with IC investigated by PCR in our molecular laboratory, concomitant viral detection, both in blood and EMB, was observed in only one EBV positive case. The frequent occurrence of negative PCR blood finding has been reported by other authors [16,17]. This is in keeping with the hypothesis that the viruses are in the blood only for a brief period followed by rapid clearance from the bloodstream, whereas false negatives may be due to poor handling of blood samples.

Among molecular biological techniques used to differentiate viral genomes [24,116,133,134], gene sequencing is the most informative allowing not only exact characterization of the infective agent but also elucidation of the molecular basis of cardiotropism as well as cardiovirulence. Genomic sites determining the virulence phenotypes are now considered the principal factors responsible for the severity of the disease. Different reports have documented that the quantity of virus recovered from the myocardium did not correlate with the extent of myocardial lesions. This is particularly true for RNA viruses, such as enteroviruses. Most of the efforts of the experts in the field, using strains engineered in the laboratory, have been principally addressed to the study of the coxsackie virus B genome [135,136]. A recent work, which used two phenotypically and genotypically distinct clinical CVB3 strains for constructing six intratypic chimeric viruses, has identified cardiovirulence determinants in the coxsackie virus B3 5’ non-translated region (5’NTR) [137].
Identification of cardiovirulent strains could provide not only important prognostic indications on the clinical progression of the disease but also useful therapeutic suggestions on responsiveness to specific medical treatment. Moreover, in the future the knowledge of genomic sites which determine the cardiovirulence could lead to the development of vaccines for patients with a major risk of lethal complications [138].

It is now equally important to have more precise etiological characterization in order to exactly define the infective status of viruses. The detection of viral replication has been demonstrated to be quite useful not only for those DNA viruses known to be in the latent form, but also for RNA viruses. Different authors have shown that the detection of CMV mRNA transcripts (early and late gene) may be considered diagnostic markers of the disease with expression of early gene preceding the antigenemia detection in most cases [139]. Both qualitative and quantitative determination of human CMV immediate-early and late (pp67) transcripts are now used for monitoring of CMV infections in the post transplantation period [140].

During viral replication of the enterovirus genome, a single-strand monocistronic RNA of positive polarity (plus-strand) acts as a template for the transcription of a replicate minus-strand RNA intermediate through the RNA-dependent RNA polymerase (3Dpol). This minus-strand RNA is then used as a template again for the 3Dpol to generate multiple copies of viral plus-strand genomes that are translated into enteroviral structural proteins and ultimately packaged into new virions. The identification of minus-strand enteroviral RNA is an indicator of active viral replication in enteroviral positive cases (positive strands). Some authors have emphasised the importance of distinguishing between active viral replication and the latent persistence of enteroviral genomes not only for prognosis with different clinical course but also for a more appropriate treatment regime [17,28,141]. A high frequency of active enteroviral RNA replication was detected not only in patients with myocarditis or clinically suspected myocarditis but also in patients with end stage DCM [17,28], where the active viral replication was associated with a poor prognosis.

Similar data are reported in HCV myocarditis where both positive and negative strands of HCV RNA were detected in cardiac tissue from patients with acute and chronic forms [43,44].

4.3.2. Monitoring of inflammatory cardiomyopathy

PCR analysis (including detection of viral replicative status and viral load) should be applied on follow-up biopsies of patients with viral myocarditis and may represent the best way to verify the efficacy of specific antiviral therapy (Fig. 3a–d) [142].

Furthermore viral persistence has been also detected by several studies that investigated the viral genome on subsequent biopsies [17,112,143]. In our recent study, three out of eight viral myocarditis cases followed by EMB merged into DC with PCR evidence of persistent viral genome and two of them died [17]. In these cases the progression into DC provides further evidence of the link between viral myocarditis and DC.

The diagnosis of viral myocarditis, which ‘per se’ is difficult particularly when based only on morphological features, becomes quite challenging in the monitoring of post-transplant endomyocardial biopsies where inflamma-
tory infiltrates within the myocardium, as a result of viral infection, are difficult to differentiate histologically from those of acute cellular rejection (Fig. 4a–c).

CMV is generally regarded as the most important infectious agent affecting organ transplant recipients, and latently infected allografts constitute a major source of the virus. The disease may result from either primary infection or reactivation of the virus in the immunosuppressed patient. More recently Towbin’s group in a large series of cases (553 consecutive biopsy samples from 149 transplant recipients) has demonstrated that different types of cardiotoxic viruses are frequently implicated[144]. In this investigation the authors stressed the crucial role of the viruses to adversely influence graft survival especially favouring chronic vascular rejection. Previous studies have shown an association between CMV infection and vascular rejection and coronary artery disease[145,146]. Different hypotheses have been postulated for the pathogenesis of allograft substrates of vasculopathy. It has been speculated that viruses may transform infected cells and thereby alter...
release of growth factors leading to cellular proliferation [147]. In addition, viruses may act directly to cause vascular endothelial damage within the heart, leading to increased expression of cell adhesion molecules and other inflammatory mediators that then promote leukocyte recruitment to the site. Viral particles, attached to the blood vessel wall, may also serve to activate the immune system, with resultant damage to the vascular endothelial surface, and viral particles may serve to alter alloantigenicity resulting in rejection [148].

De novo viral infection is not surprising in heart transplant recipients, given the immunocompromised status, however the possibility of recurrence of viral myocarditis, favoured also by the immunosuppression regimen, should be considered as well (Table 3) [17].

The demonstration of viral disease recurrence in all cardiac grafts after cardiac transplantation stresses the importance of etiologic characterization of myocarditis at the time of transplantation. Strict surveillance of these patients using molecular techniques after transplantation is mandatory for early detection of the recurrent myocarditis and consequently administration of more specific treatment, including antiviral therapy.

5. Conclusion

IC, either as acute myocarditis or chronic myocarditis/DC, is frequently triggered and often maintained by viral infection. To date morphologic studies, including immunohistochemistry for inflammatory cell characterization, associated with molecular analysis for viral detection, make EMB the gold standard for final diagnosis of the disease.

Various molecular techniques such as PCR, gene sequencing and real time PCR, often applied on the same endomyocardial specimen, have become an essential part of the diagnostic armamentarium for rapid, specific and sensitive identification of infective agents. The advantages of PCR analysis for definitive diagnosis of viral inflammatory cardiomyopathy can be summarized as follows: (i) rapid detection of specific microbial nucleic acid sequences in minute quantities; (ii) detection of agents that are difficult to cultivate or which cannot be cultivated; (iii) detection of latent and active infections; (iv) strain typing; and (v) detection of virulence and antimicrobial resistance determinants.

Correct application of molecular techniques will allow increasingly more information to be obtained: new epidemiology, current patient risk stratification and overall more appropriate medical treatment.

The high cost of biopsy processing including molecular investigation with an apparently unfavourable cost:benefit ratio, is counterbalanced by the current high morbidity and mortality (56% of patients die within 4.3 years of diagnosis) calling for precise etiologic diagnosis with inherent therapeutic implications.

William Osler, acutely ahead of his time, stated: “There are three phases to treatment: diagnosis, diagnosis and diagnosis”, stressing that the best patient management resides fundamentally in the most accurate diagnosis provided by expertise and experience [149].

References


[8] Hauck AJ, Kearney DL, Edwards WD. Evaluation of postmortem endomyocardial biopsy specimens from 38 patients with...


[124] Li Y, Bourlet T, Andreletti L et al. Enteroviral capsid protein VP1 is present in myocardial tissue from some patients with myocarditis or dilated cardiomyopathy. Circulation 2000;101:231–234.


