

Synergy of molecular and serological methods in minimally invasive diagnosis of enteroviral cardiac infection

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SUMMARY

Treatment of myocarditis and pericarditis can differ on the basis of aetiology: systemic or auto-immune disease can be positively influenced by corticoid therapy, whereas this kind of treatment can worsen the course of virus-induced disease. Therefore, the aetiological diagnosis is extremely important. The synergistic use of minimally invasive serological, IgG, IgM, IgA, and neutralizing titres, and RNA detection was evaluated on representative patients out of 238 suffering from cardiopathies. The results obtained for each case can yield reliable guidelines that rapidly highlight the presence of a viral aetiology so that an endomyocardial biopsy can be performed thus eliminating incorrect therapies. Thus, not only is this technique rapid, minimally invasive providing the clinician with decisive data, but it is cost effective for the health system.

KEY WORDS: Heart diseases, Coxsackie B3 virus, Molecular diagnosis, Neutralization assay, IFA

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INTRODUCTION

Coxsackievirus and echovirus are common worldwide infections and their prevalence is usually higher in developing countries and in temperate regions because of their fecal-oral transmission (Pallansch and Roos, 2001).

Human infection occurs either indirectly, such as contaminated food, water or environment, or directly such as the respiratory route (Pallansch and Roos, 2001).

The role of enteroviruses in cardiopathies, particularly in myocarditis and pericarditis, is known

on the basis of studies run in the 1970s and 1980s (Smith, 1970; Sainani *et al.*, 1968; Sainani *et al.*, 1975; Cambridge *et al.*, 1979). Enteroviruses infect mainly through the faecal-oral or respiratory routes. After the first localization and replication in oral-pharyngeal or intestinal mucosa, the virus is drained into deep cervical and mesenteric lymph nodes and spreads to various targets such as the central nervous system, liver, muscles, pancreas, and heart by the lympho-hematic circulation (Pallansch and Roos, 2001). After a short period of remission, a second acute episode is caused by the localization of the virus in different target organs, following the viremic phase (Pallansch and Roos, 2001).

Moreover, after the primary infection the virus can achieve persistence in myocytes (Zhang *et al.*, 2004) and potentially leads to relapses of the disease even years later. It is still not clear if corticosteroid therapy could play a role in facilitating the induction of persistence (Frustaci *et al.*, 2003).

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Even if this kind of therapy can be useful in patients with systemic or auto-immune myocarditis, its use in viral myocarditis can worsen the disease (Mason *et al.*, 1995).

To understand the true role of these viruses as aetiological agents of cardiac diseases it would be important to detect the virus in cardiomyocytes coming from endomyocardial biopsies (EMB) (Zhang *et al.*, 2004). Such an invasive sampling is obviously rarely used and is suggested only in patients with acute or fulminant heart failure of unknown aetiology or in patients unresponsive to conventional heart failure therapy (Cooper *et al.*, 2007).

Due to the limitations of the use of invasive sampling, the aetiological role of these viruses might be underestimated and the etiological diagnosis might be difficult. In this respect, less invasive methods that strongly suggest a viral aetiology can be extremely useful to reduce the number of EMB in those cases of suspected viral infection in order to confirm the diagnosis.

Detection of viral genomes in the blood stream could be a useful diagnostic tool (Andréoletti *et al.*, 2009), but viruses such as enteroviruses can persist in blood for some days and, as mentioned, there could be two viremic phases. Serology alone is rarely helpful (Cooper *et al.* 2007) both because of the high seroprevalence to enterovirus in certain populations and because of the short persistence of IgM in serum after primary infection (Dennert *et al.*, 2008).

Synergistic use of various minimally invasive diagnostic methods to detect Coxsackievirus infection in cardiac diseases is described. Their potentialities are evaluated by the description and the analysis of results obtained in some cases extrapolated as representative from the routine diagnostics for myocardial and pericardial diseases performed in our laboratory over three years. The use of this synergistic diagnosis could be useful to identify whether there is the need to confirm the diagnosis by EMB.

MATERIALS AND METHODS

Specimens

As representative cases, five of the 238 subjects suffering from cardiopathies were selected: Clinical and echocardiographic diagnosis of my-

ocarditis was suspected in 86 subjects, pericarditis in 148 and myopericarditis in four.

All the cases described were subjects suspected of suffering from cardiac diseases due to viral infection in which the diagnosis was made by serological and molecular techniques leading to one of following situations:

- a) primary infection and reactivation in a paediatric case;
- b) primary infection in an adult;
- c) myopericarditis, early diagnosis in reinfection;
- d) pericarditis, late diagnosis in reinfection;
- e) CoxB3 non infected subject.

Altogether, 20 sera were obtained, four from each of three patients, five and three from the other two patients: the first sample was taken the day of diagnosis, the second after four to six weeks, the third and the fourth at three months and not earlier than four months later respectively. All serum samples, except one, were taken and maintained in sterility for molecular diagnostic purpose, divided into aliquots and stored in DNase/RNase free tubes at -20°C until used.

Cells and virus

Coxsackie B3 (Cox B3), Nancy strain, was propagated in LLC-MK2 cells maintained in Earle's Minimum Essential Medium, supplemented with antibiotics, amphotericin B, and 2% foetal calf serum (maintenance medium). The cultures were incubated at 37°C in 5% CO₂ moist atmosphere.

Immunofluorescence assay

The tests of indirect immunofluorescence (IFA) for the detection of IgG, IgM, and IgA were carried out with minor modifications to the method described by El Falaky *et al.* (1977). Briefly, LLC-MK2 cells infected with Cox B3 were collected by scraping when a 50-70% CPE was observed. The cells were washed twice in cold PBS and 5 µl of an appropriate concentration were placed in each well of a 10 well immunofluorescence slide (bioMérieux Italia, Rome, Italy). Slides were dried at room temperature, acetone fixed at 4°C for 5 min and stored at -80° until used.

Sera were diluted twofold in PBS-0.1% Tween 20 (PBS-T) from 1:16 to 1:256 for IgG, and 1:8 to 1:64 for IgM and IgA. Ten microliters of each dilution were spotted onto the wells prepared as described above. The slides were incubated 30 min at 37°C in moist atmosphere and were then

washed three times in PBS-T. Fluorescein conjugated rabbit immunoglobulins to human IgG, IgM, or IgA (DAKO A/S, Denmark) were diluted 1:100, 1:40, and 1:20, respectively, in PBS-T supplemented with Evans Blue as counterstain. Ten microliters of diluted conjugates were spotted on each well and the slides were again incubated for 30 min as above. After washing, the slides were dried and then observed under an epifluorescence microscope.

Microneutralization test

LLC-MK2 cells were grown in flat bottomed 96 well polystyrene microplates.

Sera to be tested were heated at 56°C for 20 min and then left to cool to room temperature (RT). They were serially diluted from 1:4 to 1:256 in maintenance medium containing 100 PFU/100 µl Cox B3, and incubated for 1 h at RT. Each serum dilution was plated in triplicate and incubated as described. The plates were observed daily until CPE was observed in the wells used as virus control. The neutralizing antibody titre was the highest serum dilution that inhibited the CPE.

Enterovirus RNA detection

Viral RNA extraction was performed by a commercial kit (QIAmp Viral RNA, QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. From 280 µl of serum 60 µl of eluted RNA was obtained.

The RNA was subjected to a seminested PCR that amplified the 5'NC region, standardized in our laboratory with minor modifications to the method described by Andréoletti *et al.* (1998).

All positive samples were retested for confirmation by a commercial real time PCR test (Enterovirus qPCR Alert Kit, Nanogen Advanced Diagnostics s.r.l., Turin, Italy) that amplified the 5'UTR region.

RESULTS

Enterovirus RNA was detected in all samples except one that did not fully respect the sterility criteria. Results are shown in table 1.

The results of each of the five cases listed above were as follows:

a) *primary infection and reactivation in a paediatric case* (Table 1; Figure 1, case a): was an 8-

TABLE 1 - Results obtained from specimens tested; antibody values are expressed as reciprocal titre.

Case	Weeks	Neutr.*	IgG	IgM	IgA	PCR
a)	0	8	128	16	32	N.T.†
	6	8	128	<8	8	Neg.
	12	128	128	<8	32	Pos.
	17	16	64	N.T.	8	Neg.
b)	0	16	64	64	32	Pos.
	4	8	64	8	8	Pos.
	12	128	32	<8	8	Neg.
	21	16	64	<8	8	Neg.
	34	8	64	<8	8	Neg.
c)	0	64	16	<8	<8	Pos.
	4	128	128	<8	16	Neg.
	12	256	256	<8	8	Neg.
	30	128	256	N.T.	8	Neg.
d)	0	128	64	<8	32	Pos.
	4	64	64	N.T.	8	Neg.
	12	128	64	N.T.	8	Neg.
	17	128	64	N.T.	8	Neg.
e)	0	16	16	<8	8	Neg.
	4	8	16	N.T.	<8	Neg.
	12	8	16	N.T.	<8	Neg.

*Microneutralization test; †Not tested

year-old girl with primary acute pericarditis. Despite the fact that the IgG titre was high, she suffered from a relapse 90 days after the first sample was taken.

The first serum sample was not suitable for the detection of RNA. Specific IgM and IgA were detectable in the first serum sample that was taken about four weeks after symptoms arose and indicated that pericarditis could be due to primary Cox B3 infection.

The neutralizing antibody titres in the first two samples were very low and allowed the virus to reactivate, leading to relapse. In the third sample, in the course of the relapse, RNA was detectable as well as a relevant increase of the titres of specific IgA and of neutralizing antibodies. These data indicate that a low titre of neutralizing antibodies facilitates the reactivation of the infection while high IgG titres detected by IFA are not always protective.

b) *primary infection in adult* (Table 1; Figure 1, case b): A primary Cox B3 infection was diagnosed in an adult patient (female, 48 y) with fib-

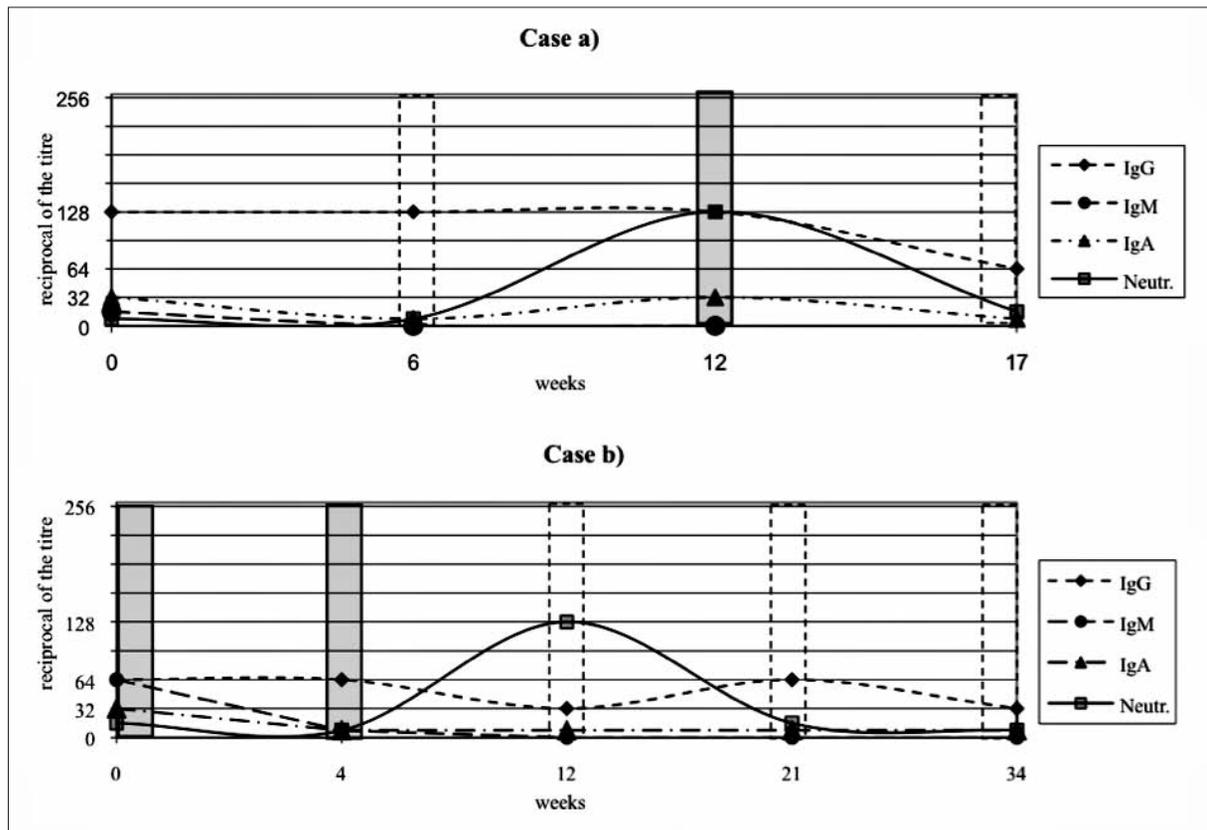


FIGURE 1 - Comparison of shape in primary infections: case a) primary infection and reactivation in a paediatric case; case b) primary infection in adult.

rinous pericarditis. Specific high titre IgM and IgA were found in the first sample obtained about seven weeks after the appearance of non-specific symptoms. Neutralizing antibodies were low in the same serum sample and enteroviral RNA was detectable in two subsequent sera probably because of the low titre of neutralizing antibodies, and IgM and IgA persisted longer than usual after recovery. RNA was not detected in the third sample, in the presence of high neutralizing antibodies while IgG detected by IFA maintained the same titre and IgM became negative. This patient, who has been in follow-up since 2002, had three symptomatic relapses in six years (data not shown). In all the acute phases she was RNA positive in the presence of a low neutralizing antibody titre that increased over the next three weeks: RNA was never detected in sera with a neutralizing titre $\geq 1:128$.

c) *myopericarditis, early diagnosis in reinfection* (Table 1; Figure 2, case c): female, 56 y with my-

opericarditis, pericardial effusion and heart failure. Specific IgM and IgA were negative in the first sample, IgG by IFA were positive at a low titre. From these results this cardiopathy might appear not to be due to a Cox B3 infection. On the other hand, the microneutralization test titre and the presence of enteroviral RNA indicated this virus as the aetiological agent of the pathology. In fact, in the following serum samples, all the immunoglobulin titres, except the IgM, increased and the RNA detection became negative. All these data, in the absence of IgM, clearly identify this case as reinfection which was rapidly diagnosed because of the early emergence of symptoms that gave the opportunity to collect an early first sample.

d) *pericarditis, late diagnosis in reinfection* (Table 1; Figure 2, case d): This case (female 59 y) with fibrinous pericarditis could appear similar to the previous one, but all the results obtained in the first sample resemble those observed in the sec-

ond sample of the previous case. This was classified as a late diagnosed Cox B3 reinfection probably because of the late appearance or feeling of symptoms as frequently happens in the course of pericarditis.

e) *Pericarditis not CoxB3 related* (Table 1): The pericardial effusion that afflicted this patient (fe-

male, 74y) was not due to Cox B3. Even if there was a low IgA titre, the low IgG titre and neutralizing antibodies, in the absence of enteroviral RNA in any sample, indicated that the aetiology of that pathology was not due to Cocksackie. It was confirmed by the results obtained in the following two serum samples.

TABLE 2 - General guidelines for interpretation of the results.

RNA	IFA				Diagnosis
	Neutr.	IgG	IgM	IgA	
+/-	Low/increasing	+	+/decreasing	+/decreasing	Primary infection
+	+/high	+/increasing	-	- to +	Re-infection E.D.*
+	+/high	+	-	+/decreasing	Re-infection L.D.**
-	+ (low)/-	+ (low)/-	-	-	No active infection

*E.D.: early diagnosis; **L.D.: late diagnosis

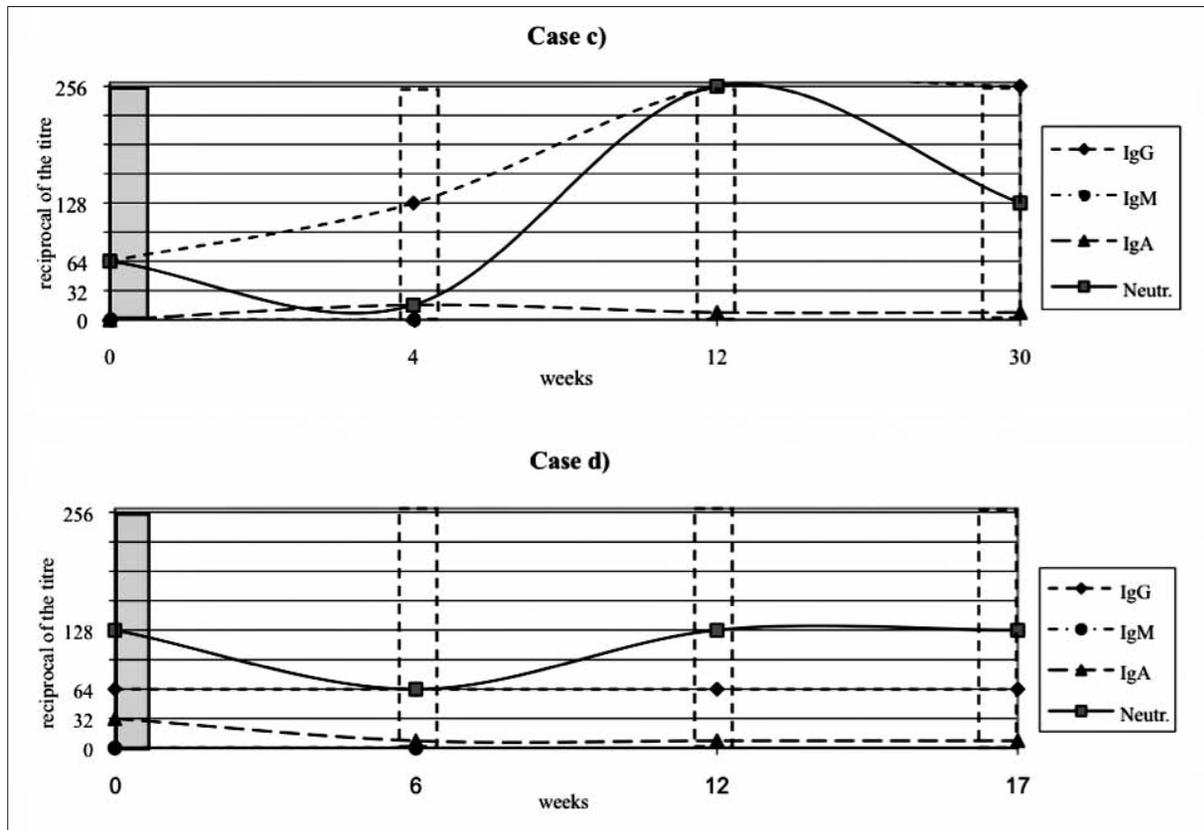


FIGURE 2 - Comparison of shape in reinfections: case c) myopericarditis, early diagnosis in reinfection; case d) pericarditis, late diagnosis in reinfection.

DISCUSSION

Coxsackievirus B is the aetiological agent of major human diseases, such as those afflicting the CNS (Berger *et al.*, 2006; Papa *et al.*, 2006; Böttner *et al.*, 2002), pancreas (Berg *et al.*, 2007; Foulis *et al.*, 2008; Scarsvik *et al.* 2006; Filippi and von Herrat, 2005) and heart. Among these, myocarditis and pericarditis are frequently reported both in adults (Maisch *et al.*, 1982; Kim *et al.*, 2001; Andréoletti *et al.*, 2000) and in children Dettmeyer *et al.*, 2002; Amabile *et al.*, 2006; Rozkovec *et al.*, 1985; Felker *et al.*, 2000a). Cardiac symptoms could arise either early or even weeks after infection with acute chest pain simulating acute myocardial infarction or breathlessness as described also in our cases. Myocarditis can be completely asymptomatic, evolving later into chronic heart failure, or can appear as acute arrhythmia sometimes leading to death (Feldman and McNamara, 2000).

Serology in viral myocarditis or pericarditis was for many years one of the few tools available for the diagnosis of Coxsackievirus infection (Cambridge *et al.*, 1979; Cooper *et al.*, 2007; Felker *et al.*, 2000b). In the past years, and sometimes even today, these cardiac pathologies are treated first by corticosteroids that alter the immunological response and make the interpretation of the serological results difficult. Thus, the use of more than one diagnostic tool appears to be useful for a more accurate diagnosis. The purpose of this study is to discuss the results obtained from five cases, chosen as examples of different conditions among those observed in routine diagnosis, in order to devise possible guidelines for the mini-invasive diagnosis of Coxsackievirus infection.

The results indicate that the use of a single diagnostic parameter cannot have a definitive diagnostic relevance. IgM are usually considered a reliable parameter for the diagnosis of primary viral infection. Currently, the role of IgM in serological diagnosis, in our opinion, is often overestimated especially in the diagnosis of enterovirus infections. For example, the decay time of these immunoglobulins in Coxsackie infection is short; negative results could be due to infections that took place four-six weeks before sampling or in endogenous or exogenous reinfections. Detection of specific IgM is more frequent and significant

in paediatric cases because children have usually not experienced a Coxsackievirus infection before, and because parents consult paediatricians soon after symptoms arise. Thus, the use of IgM titration might be considered an important tool for the diagnosis of Coxsackievirus especially in primary infection in children.

As concerns the neutralizing antibodies, our results indicate their primary diagnostic relevance even if their titration is elaborate and time-consuming. In fact, the increasing titre of these antibodies can be observed during or shortly before the viremic phase and a low neutralizing titre can prelude or predispose to a potential reactivation and/or relapse. Thus, this method should be reconsidered as important both for the diagnosis and for the follow-up of patients.

The analysis of the results obtained in the five cases described above focuses on the significance of each of the three diagnostic parameters that are rarely employed in routine diagnosis: IgA, enteroviral RNA, and neutralizing antibodies.

An increase in IgA titres can be indicative of recent either primary infection or endogenous or exogenous reinfection. Then, when IgM are negative, IgA titres $\geq 1:16$ can be considered indicative of possible reinfection that otherwise could be difficult to diagnose. The cut-off titre 1:16 was calculated following the suggestions of Guglielmino *et al.* (1989) on the basis of the statistical inference coming from the analysis of the IgA titre distribution routinely detected in the laboratory from healthy subjects (unpublished data).

Enteroviral RNA detection in the bloodstream is an important tool for the diagnosis of this kind of infection (Andréoletti *et al.*, 2009). Unfortunately it does not always coincide with the acute phase of the pathology: sometimes it becomes detectable only weeks after the humoral immune response. This could appear a limitation for its diagnostic relevance. If the pathogenesis of enteroviral infections is taken into consideration, it appears evident that there is a bimodal infection course: first, minor viremia with systemic reticuloendothelial tissue infection following the first localization at the site of entry, and second, major viremia that leads to localization to the target organs (Pallansch and Roos, 2001).

The observation and the analysis of the results obtained lead to the hypothesis that the time of the Coxsackie infection, at least as regards peri-

carditis, does not always coincide with the presence of the virus in the blood stream, but can precede this phase.

Therefore, viremia, during cardiac replication, should be minimal or absent but the humoral immune response is already active and specific antibodies are usually detectable. In the course of the second viremia, the virus can be detectable in the bloodstream and persist for some days. In this phase serology response does not give significant information since the immune response is activated during the first phase of infection. The results obtained by serological and molecular tests, combined with the clinical data, can give a more definite diagnosis that can indicate if the cardiac disease observed has Cox B3 as its aetiological agent (Table 2).

This can lead to the identification of highly probable enteroviral infection cases and, then, to a potential reduction of invasive diagnostic procedures that, however, remain the gold standard for the definitive diagnosis (El Falaky *et al.*, 1977). Thus, not only is this technique rapid and minimally invasive providing the clinician with decisive data for a possible invasive biopsy, but it is cost effective for the health system.

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