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from the N terminus of the VP1 protein of coxsackievirus B4. Sera positive for IgG antibodies against coxsackieviruses B1/B5, B3, and B4 were detected in 6.7 to 21.6% of the individuals tested in the various regions of Greece. Statistical analysis revealed that the highest prevalence of IgG antibodies against coxsackieviruses B1/B5 was found in blood donors from Crete ($p = 0.025$), whereas the highest prevalence against coxsackievirus B4 was detected in blood donors from Athens ($p = 0.01$). IgM antibodies against coxsackievirus B were detected at low percentage, less than 5%, with no significant viral preference for particular geographic regions. The preference of anti-coxsackievirus IgG antibodies for particular geographic regions could be potentially related to the previously reported clustering of cases of insulin-dependent diabetes mellitus and myocarditis in Athens and Crete, respectively.

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Serologic Prevalence of Coxsackievirus Group B in Greece

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ABSTRACT

Coxsackieviruses are human enteroviruses, which have been associated with myocarditis/pericarditis and sudden death. In one investigation (Spanakis N, Manolis EN, Tsakris A, Tsiodras S, Panagiotopoulos T, Saroglou G, and Legakis NJ: J Clin Pathol 2005;58:357–360), a cluster of cases of fatal myocarditis in Greece was linked to coxsackievirus B3. The information from this investigation prompted us to study serologically the prevalence of coxsackieviruses B throughout Greece. Sera were obtained from 506 healthy blood donors from various transfusion centers, covering the entire country. All sera were tested for the presence of IgG and IgM antibodies, using ELISAs with various antigenic specificities: (1) heat-denatured coxsackievirus type B1 and B5 virions, (2) a synthetic peptide from the N terminus of the VP1 protein of coxsackievirus B3, and (3) a synthetic peptide from the N terminus of the VP1 protein of coxsackievirus B4. Sera positive for IgG antibodies against coxsackieviruses B1/B5, B3, and B4 were detected in 6.7 to 21.6% of the individuals tested in the various regions of Greece. Statistical analysis revealed that the highest prevalence of IgG antibodies against coxsackieviruses B1/B5 was found in blood donors from Crete ($p = 0.025$), whereas the highest prevalence against coxsackievirus B4 was detected in blood donors from Athens ($p = 0.01$). IgM antibodies against coxsackievirus B were detected at low percentage, less than 5%, with no significant viral preference for particular geographic regions. The preference of anti-coxsackievirus IgG antibodies for particular geographic regions could be potentially related to the previously reported clustering of cases of insulin-dependent diabetes mellitus and myocarditis in Athens and Crete, respectively.

INTRODUCTION

COXSACKIEVIRUSES ARE HUMAN ENTEROVIRUSES belonging to the family *Picornaviridae*. They are further subdivided into two serogroups, A and B, which comprise 23 (1–22 and 24) and 6 (1–6) serotypes, respectively. Coxsackieviruses are transmitted primarily via the fecal–oral route and as respiratory aerosols. They replicate in the oropharyngeal and intestinal epithelium and, from there, they can be carried by the blood stream

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to the cells of the reticuloendothelial system and certain target tissues or organs such as the pancreas, liver, myocardium, meninges, and skin. More than 90% of these infections progress subclinically and they rarely cause life-threatening illness (26,27). Coxsackieviruses A have been associated with mild clinical syndromes such as mild flu-like illness (e.g., summer flu) as well as with conditions such as outbreaks of aseptic meningitis, whereas coxsackieviruses B are responsible for pancreatitis, hepatitis, aseptic meningitis, and myocarditis/pericarditis (5,14,25,27).

Viral infections of the heart are important causes of morbidity and mortality in all age groups without sex predominance. Coxsackieviruses B, especially CVB3 and CVB5 (15,17,19), are the most common causes of viral myocarditis and may be detected in more than 25% of sporadic cases of acute onset of dilated cardiopathy (8,12,18), whereas coxsackievirus B1 is mentioned as a rare cause (16). In addition, coxsackievirus B5 is frequently associated with sporadic cases of neurological diseases and epidemics of meningitis (21). Coxsackievirus B4 has long been implicated in the development of insulin-dependent diabetes mellitus (IDDM) (11,22), but there are studies indicating that several other serotypes of coxsackieviruses B, such as B1, B2, B3, and B5, might play a role in the pathogenesis of this chronic disease (2,22).

Among others, climate appears to be an important factor in the circulation and prevalence of enteroviruses in temperate regions. Enteroviruses are generally present at low levels in the winter and spring, but are isolated far more commonly during the summer and fall. Even in the United States, healthy children in the southern cities harbor a greater abundance of enteroviruses than do those of comparable age in the northern cities (7).

In one report, a nationwide investigation of a cluster of cases of acute respiratory tract syndrome associated with myocarditis and/or pericarditis took place in Greece, from January to April 2002. This study indicated that there was a link between enteroviruses, especially coxsackievirus B3, and direct tissue damage in three fatal myocarditis cases in Crete (24).

The information in this report prompted us to study serologically the prevalence of coxsackieviruses B throughout Greece, to determine whether there is a viral preference for particular geographic regions.

MATERIALS AND METHODS

Sera

Sera were obtained from 506 healthy blood donors, aged between 25 and 35 yr, from different collection sites throughout Greece. Collection sites were selected to cover the entire country, including northern (Thessaloniki), southern (Iraklio/Crete), western (Ioannina and Patra), and eastern (Larisa and Athens) Greece (Fig. 1). The samples were collected during the summer of 2005 (from June 1 to August 31) and the ratio of male to female was about 1:1. All sera were tested for the presence of IgG and IgM antibodies by enzyme-linked immunosorbent assay (ELISA) with different antigenic specificity: (a) heat-denatured coxsackie type B1 and B5 virions, (b) a synthetic peptide representing the N terminus of the VP1 protein of coxsackievirus B3 (peptide CVB3), and (c) a synthetic peptide representing the N terminus of the VP1 protein of coxsackievirus B4 (peptide CVB4).

Synthetic peptides

Amino acid sequences of the major VP1 antigen of coxsackieviruses were compared between different serotypes of coxsackievirus B species. Differences were observed mainly in the amino-terminal region of the VP1 antigen, a region previously defined to hold B cell antigenic determinants, as determined by epitope-mapping experiments. Therefore, two peptides spanning amino-terminal region 1–15 of coxsackievirus B3 (GPVEDAITAAIGRVA) and coxsackievirus B4 (GPTEESVERAMGRVA) were synthesized by solid-phase peptide synthesis (Bio-Synthesis, Lewisville, TX). The peptides were purified by high-pres-

FIG. 1. Sera were obtained from 506 healthy blood donors from various hospitals of northern (Thessaloniki), southern (Iraklio/Crete), western (Ioannina and Patra), and eastern (Larisa and Athens) Greece.

sure liquid chromatography (HPLC) and their identity and purity were confirmed by mass spectroscopy (MS).

ELISA for detection of antibodies specific to coxsackieviruses B3 and B4

Preliminary ELISA experiments were performed in order to define the optimal conditions. The optimal peptide concentration for coating was determined to be 10 μg/mL for peptide CVB3 and 5 μg/mL for peptide CVB4. ELISA plates (Costar; Corning Life Sciences, Acton, MA) were coated overnight at 4°C with the peptides diluted in phosphate-buffered saline (PBS, pH 7.2) at their optimal concentrations. Afterward, the plates were blocked with, per well, 200 μL of bovine serum albumin (BSA), 2% in PBS (pH 7.2), for 1 h at room temperature. The plates were incubated overnight at 4°C with human serum diluted 1:200 in blocking buffer. After three washes with PBS, alkaline phosphatase-conjugated anti-human IgG or IgM (Jackson ImmunoResearch, West Grove, PA) (diluted 1:1100 in blocking buffer) was added in order to detect antibodies bound onto the solid phase. After an incubation period of 1 h at room temperature, the plates were washed three times with PBS and the enzyme reaction was developed with *p*-nitrophenyl phosphate substrate (Sigma, St. Louis, MO). The optical densities (ODs) were quantified at 405 nm with an ELISA reader (Bio-Tek Instruments, Winooski, VT). To normalize our OD readings between different ELISA plates, three common positive sera and three common normal sera were used as counterbalancing controls in each plate. Experiments with OD coefficient variation more than 10% were repeated. All ODs were transformed and expressed as binding units according to the following formula: binding units (BU) = $(OD_{Sample}/OD_{PosCtrl}) \times 100$, where OD_{Sample} is the OD reading of the current sample and $OD_{PosCtrl}$ is the mean OD of the three positive controls in the current ELISA plate. The cutoff value for anti-peptide ELISA was calculated as mean normal serum binding units plus 3 standard deviations. Samples were considered positive when the corrected OD was above the cutoff.

ELISA for detection of antibodies specific to coxsackieviruses B1 and B5

For the detection of specific antibodies against coxsackieviruses B1 and B5, we used a commercially available assay (SERION ELISA *classic* coxsackievirus; Serion Immunodiagnostica, Würzburg, Germany), using heat-denatured coxsackievirus type B1 and B5 virions as the antigen source. In brief, 100 μL of patient serum (diluted 1:800) was added to the wells, precoated with heat-treated coxsackievirus type B1 and B5 virions, and was incubated for 1 h at 37°C in a moist chamber. For the IgM ELISA, the serum samples were preincubated with rheumatoid factor removal reagent (SERION ELISA *classic*; Serion Immunodiagnostica) to prevent false positives due to potential rheumatoid factor binding to IgG antibodies. After removal of unbound material by washing, 100 μL of anti-human IgG or IgM antibody conjugated to alkaline phosphatase was allowed to react with the immune complex for 30 min at 37°C, in a moist chamber. After removal of excess conjugate, *p*-nitrophenyl phosphate substrate was added and the color was developed. The enzymatic reaction was stopped by addition of 100 μL of 1.2 M sodium hydroxide. The OD was quantified photometrically at 405 nm. The ODs were converted to binding units (BU), using a specific formula provided by the manufacturer, based on the measured OD of the control serum samples. According to the manufacturer's instructions for the IgG and IgM ELISAs, respectively, results of $>$100 and $>$50 BU/mL were considered positive, results of 80–100 and 30–50 BU/mL were regarded as borderline, and results less than 80 and 30 BU/mL were considered negative. Samples giving borderline results were characterized as doubtful.

Statistical analysis

Using the χ^2 test, statistical comparisons between seroprevalence rates were performed according to the sampling area.

RESULTS

Prevalence of IgM antibodies against coxsackieviruses B1/B5, B3, and B4

We aimed to study the prevalence of anti-coxsackievirus antibodies on the basis of two distinct ELISA systems, one a commercial assay for the detection of anti-CVB1/B5 antibodies and the other an in-house ELISA (based on synthetic epitopes) for the detection of anti-CVB3 and anti-CVB4 antibodies. For the vast majority of sera, ELISAs for the detection of IgM antibodies against coxsackieviruses B1/B5 were negative (titer, $<$30 BU/mL) (Fig. 2a). Borderline levels of IgM antibodies (titer, 30–50 BU/mL) were detected in 9.7, 6.4, 3.2, 3.3, 3.2, and 3.3% of blood donors from Athens, Thessaloniki, Ioannina, Iraklio, Larisa, and Patra, respectively. The only geographic region that gave a positive result for antibodies against coxsackieviruses B1/B5 (titer, $>$50 BU/mL) was Iraklio, with an IgM prevalence of about 3.3%.

IgM antibodies against coxsackievirus B3 were detected in 3.3, 6.5, 6.9, and 3.1% of the individuals tested from Athens, Thessaloniki, Iraklio, and Patra, respectively (Fig. 2b). In parallel, IgM antibodies against coxsackievirus B4 were found in 3.1% of the blood donors

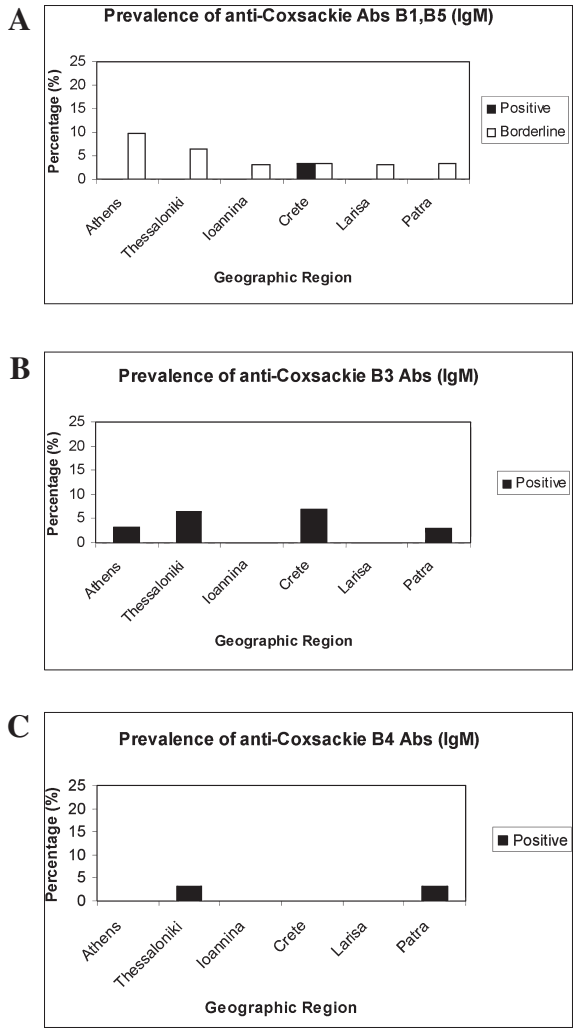


FIG. 2. Prevalence of IgM antibodies against coxsackie group B viruses. (**a**) Anti-CVB1/CVB5 IgM antibodies. Borderline levels of IgM antibodies against coxsackieviruses B1 and B5 were calculated according to the manufacturer's instructions and are presented as open columns. (**b**) Anti-CVB3 IgM antibodies (VP1 peptide assay); (**c**) anti-CVB4 IgM antibodies (VP1 peptide assay).

from Thessaloniki and Patra (Fig. 2c). Statistical analysis of the data revealed that there was no significant viral preference for particular geographic regions.

Prevalence of IgG antibodies against coxsackieviruses B1/B5, B3, and B4

Sera positive for IgG antibodies (>100 BU/mL) against coxsackieviruses B1 and B5 were detected in 18.5, 16.3, 10.9, 17.4, 12, and 15.2% of the individuals tested from Athens, Thessaloniki, Ioannina, Iraklio, Larisa, and Patra, respectively. Borderline results (titer, 80–100 BU/mL) were obtained for 6.5, 7.6, 9.8, 17.4, 7.6, and 15.2% of the serum samples from Athens, Thessaloniki, Ioannina, Iraklio, Larisa, and Patra, respectively (Fig. 3a). Overall, the prevalence of IgG antibodies against coxsackieviruses B1 and B5, including positive and borderline results (cutoff, 80 BU/mL), was found to be significantly higher in blood donors from Iraklio ($p = 0.025$).

IgG antibodies against synthetic peptide representing the N terminus of the VP1 protein of coxsackievirus B3 were detected in the serum of 21.8, 17, 21.6, 17.4, 12.2, and 15.7% of blood donors from Athens, Thessaloniki, Ioannina, Iraklio, Larisa, and Patra, respectively (Fig. 3b). In comparing the prevalence of IgG antibodies against coxsackievirus B3 amongst different geographic regions, no statistically significant difference was seen.

Similarly, IgG antibodies against synthetic peptide representing the N terminus of the VP1 protein of coxsackievirus B4 were found in 19.1, 13.6, 9.3, 11.6, 11.1, and 6.7% of the serum samples from Athens, Thessaloniki, Ioannina, Iraklio, Larisa, and Patra, respectively (Fig. 3c). The highest prevalence of IgG antibodies against coxsackievirus B4 was detected in blood donors from Athens ($p = 0.01$).

Correlation of IgG antibody titers against different coxsackievirus B subtypes

To examine the possibility of detecting, by peptide ELISA, antibodies cross-reactive to different coxsackievirus B subtypes, we correlated antibody titers in the different assays used (Fig. 4). We found no correlation between either anti-CVB4 or anti-CVB3 with anti-CVB1/CVB5 reactivities. Therefore, the assays used for the detection of anti-CVB3 and anti-CVB4 antibodies were specific, because there was a clear discrimination of reactivities between anti-CVB1/B5-positive sera and sera positive for either of the two peptides (pepCVB3 or pepCVB4). Thus, although CVB3 and CVB4 amino-terminal peptides of VP1 share a significant degree of sequence similarity with the homologous CVB1 and CVB5 peptides of VP1 (Table 1), the potential cross-reactivities are limited to a small subpopulation of the tested sera.

DISCUSSION

Most coxsackievirus B infections, probably about 90%, are subclinical, or may lead to only mild diseases (26). In the remaining cases the outcome can be serious, and even fatal. Furthermore, whereas many of these diseases follow an acute course, some others may be chronic, inducing long-term health problems. Coxsackievirus B is the commonest infectious cause of myocarditis and dilated cardiomyopathy (14), which in the Western world

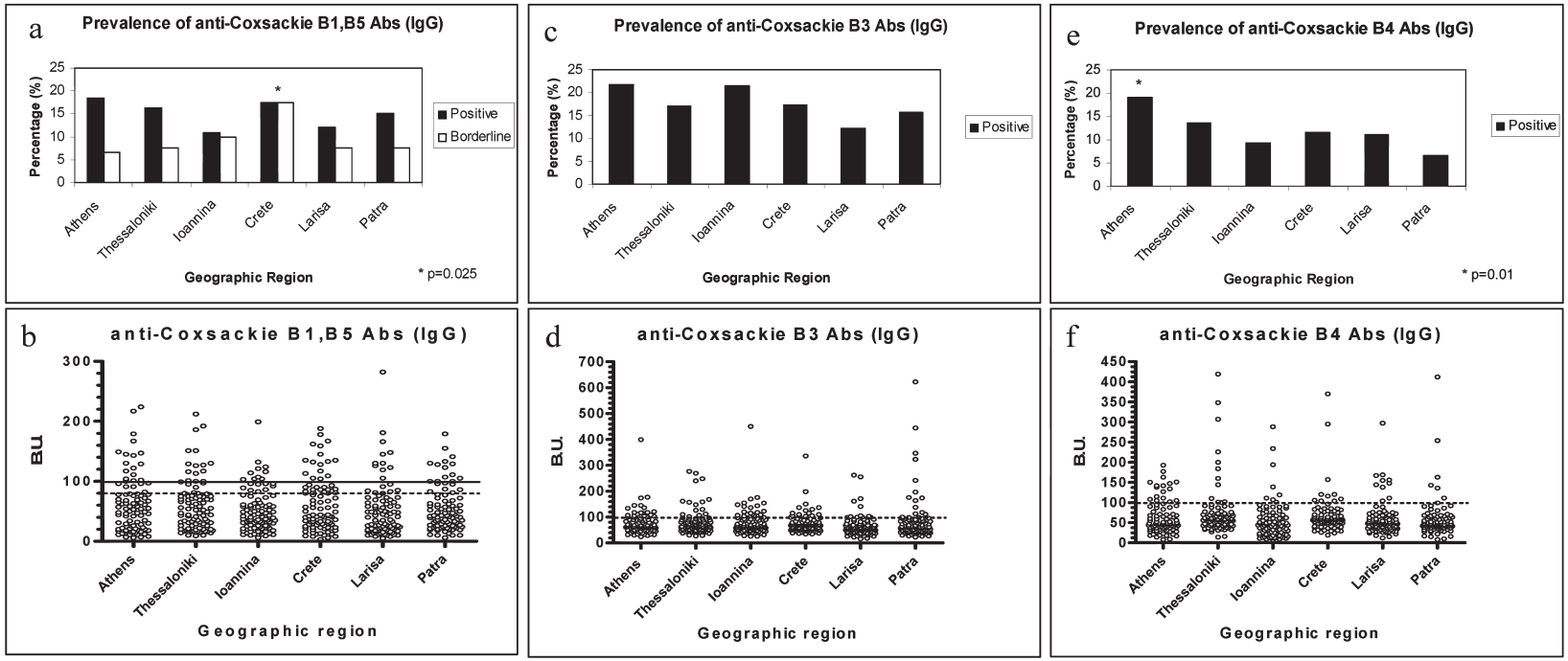
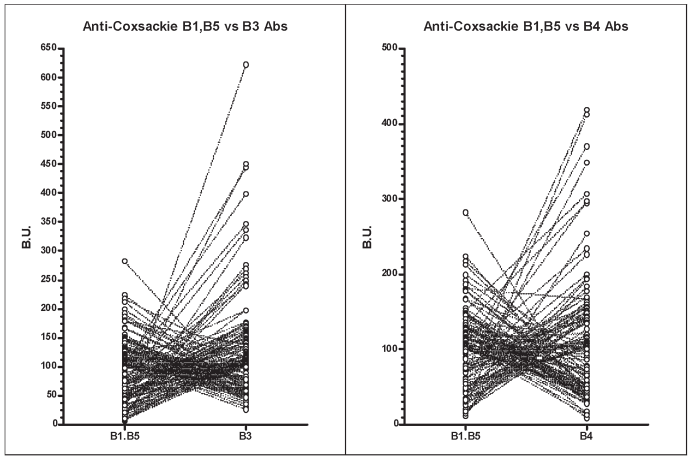


FIG. 3. Prevalence of IgG antibodies against coxsackie group B viruses. (**a**) Prevalence of anti-CVB1/CVB5 IgG antibodies. Borderline levels of IgG antibodies against coxsackieviruses B1 and B5 were calculated according to the manufacturer's instructions and are presented as open columns. (**b**) Optical density (OD) of anti-CVB1/CVB5 IgG antibodies (VP1 peptide assay). (**c**) Prevalence of anti-CVB3 IgG antibodies (VP1 peptide assay). (**d**) Optical density of anti-CVB3 IgG antibodies (VP1 peptide assay). (**e**) Prevalence of anti-CVB4 IgG antibodies (VP1 peptide assay). (**f**) Optical density of anti-CVB4 IgG antibodies (VP1 peptide assay). Dotted lines represent the cutoff values.



anti-Coxsackie B3

anti-Coxsackie B1,B5	(+)	(-)	
(+)	9	60	69
(-)	76	361	437
	85	421	506

chi-square=0.81, NS

anti-Coxsackie B4

anti-Coxsackie B1,B5	(+)	(-)	
(+)	9	60	69
(-)	51	386	437
	60	446	506

chi-square=0.11, NS

FIG. 4. Correlation of IgG antibody titers between coxsackieviruses B1 and B5 and the coxsackievirus B3 or B4 subtype. The reactivities of anti-CVB3 or anti-CVB4 and anti-CVB1/CVB5 IgG antibodies were not found to correlate significantly.

is the underlying etiology in about 45% of patients undergoing heart transplantation (10,13).

Evidence of enteroviral infection among a cluster of fatal myocarditis cases was found in the Greek island Crete from January to April 2002 (24). This study indicated that there was a link between coxsackievirus (especially the B3 serotype), and direct myocardial damage. Crete is located in southern Greece, where high temperatures last longer than in other areas of the country. It is well known that the circulation of enteroviruses is increased in temperate climates, because enteroviral infections occur most frequently in southern regions of the United States (7). Moreover, resolution of the pathways of enteroviruses during outbreaks revealed that enteroviral circulation is apparently localized to southern communities during the winter, spreading north during the summer (23).

The main aim of this study was to define the serologic prevalence of coxsackie group B viruses in different regions of Greece, covering the entire country (Fig. 1). Because of the unavailability of commercial immunoassays for the detection of antibodies against the CVB3 and CVB4 subtypes, specific ELISAs based on VP1 amino-terminal peptides were developed.

TABLE 1. SEQUENCE SIMILARITY BETWEEN AMINO-TERMINAL PEPTIDES OF VARIOUS COXSACKIEVIRUS SUBTYPES

N-terminal peptide, VP1 protein	*Amino acid sequence*
CVB1	GPV**E**ESVER**A**MVRA
CVB3	GPVEDAITA**A**IGRVA
CVB4	GPTEESVER**A**MGRVA
CVB5	G**P**PGEAVER**A**IA**RV**A

Borderline IgM antibodies against coxsackieviruses B1 and B5 were detected in 3.2–9.7% of serum samples from different regions of Greece. The only geographic region that gave positive results, with an IgM prevalence of about 3.3%, was Iraklio/Crete (Fig. 2a). IgM antibodies against coxsackievirus B3 or B4 exhibited no significant preference for particular geographic regions (as shown in Fig. 2b and c). Although a positive IgM test result indicates an acute or recent coxsackieviral infection, persisting IgM antibodies can be found in about one-third of patients with myocarditis and in about two-thirds of patients with aseptic meningitis. Persisting IgM antibodies (>6 mo) may also indicate chronic diseases such as chronic fatigue syndrome, relapsing pericarditis, or type 1 diabetes (20).

IgG antibodies against coxsackieviruses B1 and B5 were detected in 10.9–18.5% of the sera tested, and borderline levels of IgG antibodies with the same specificity were found in 6.5–15.2% of the sera (Fig. 3a). The prevalence of coxsackievirus B1/B5 IgG antibodies, including positive and borderline results, was found to be significantly ($p = 0.025$) higher among blood donors from Iraklio/Crete compared with donors from other collection sites.

Analysis of data showed that 15.7–21.8% of sera from Greek blood donors had IgG antibodies against the synthetic peptide representing the terminus of the VP1 protein of coxsackievirus B3, without any statistically significant preference for a geographic region (including Iraklio/Crete) (Fig. 3b). We should note that all six CVB serotypes induce direct, irreversible toxicity toward cardiomyocytes, which eventually leads to the death of infected cells. All the CVB viruses lead to direct irreversible cardiomyocyte toxicity, regardless of CVB serotype (1).

IgG reactivity against synthetic peptide representing the N terminus of the VP1 protein of coxsackievirus B4 was observed in 6.7–19.1% of Greek serum samples (Fig. 3c). The highest prevalence of IgG antibodies against coxsackievirus B4 was detected in blood donors from Athens ($p = 0.01$). There is strong epidemiologic, serologic, and molecular evidence associating coxsackievirus B4 infection with the development of type 1 diabetes mellitus in humans (11).

It is notable that the incidence of IDDM in the Athens region was found, in one study, to be greater (10 per 100,000 children) than in five other regions of northern Greece (fewer than 5 per 100,000) (3). This is almost twice the incidence of IDDM in the countryside (mixed urban and rural) population of northern Greece (6). Our data are consistent with this, with anti-CVB4 positive sera clustered in the Athens metropolitan area, possibly reflecting the role of coxsackieviruses in the development of IDDM (4,9).

Concerning the specificity of the peptide ELISAs for the detection of anti-CVB3 and anti-CVB4 antibodies, we found no correlation of antibody titers measured in anti-CVB4 and anti-CVB3 assays with those measured in commercial anti-CVB1/CVB5 ELISAs (Fig. 4). The vast majority of the anti-CVB1/CVB5-positive sera (87.0%) did not give positive OD readings in either anti-CVB3 or anti-CVB4 ELISA. These data suggest that the peptide ELISAs, used in our study, were specific for the detection of antibodies against different coxsackievirus subtypes.

In conclusion, the prevalence of antibodies against different coxsackievirus B subtypes in Greece ranges from 9.3 to 21.8%, with antibodies against coxsackievirus B4 detected to a greater extent in Athens and antibodies against coxsackievirus B1/B5 in Iraklio/Crete. This preference for particular geographic regions must be investigated, considering the previously reported clustering of cases of IDDM and myocarditis in Athens and Crete, respectively.

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