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Comparison of Coxsackie B Neutralisation and Enteroviral PCR in Chronic Fatigue Patients

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Coxsackie B enteroviruses have been implicated repeatedly as agents associated with chronic fatigue syndrome (CFS). The objective of this study was to compare the serological evidence for the presence of Coxsackie B virus neutralising antibody, with the polymerase chain reaction (PCR) detecting a portion of the 5′ nontranslated region (NTR) of the enterovirus genome. Serum samples from 100 chronic fatigue patients and from 100 healthy comparison patients were used in this study. In the CFS study group, 42% patients were positive for enteroviral sequences by PCR, compared to only 9% of the comparison group. Using the neutralisation assay, 34% of study patients were positive, compared to 41% of comparison patients. In the study group, 66/100 patient results correlated, i.e., they were either positive/positive or negative/negative for both tests. Of those that did not correlate, the majority were PCR-positive/Coxsackie B antibody-negative (21/34). In the comparison group, 58/100 patient results correlated. Of those that did not, the majority were PCR-negative/Coxsackie B antibody-positive (37/42). The Coxsackie B antibody neutralisation assay was not able to differentiate the CFS study group from the healthy comparison group, and thus the clinical relevance of this assay may be questioned. The PCR assay did differentiate the two groups with significantly more CFS patients having evidence of enterovirus than the comparison group.

KEY WORDS: serology, nucleic acid, virus detection, diagnostic detection

INTRODUCTION

Enteroviruses and in particular Coxsackie B viruses can cause a wide variety of human diseases as well as being involved in asymptomatic or minor infections [Minor and Bell, 1990]. In individuals with intact immunity, enteroviral infection is considered to follow a self-limiting course. However, it has now been recognised that persistence can occur in experimental systems and also perhaps in humans, with recent reports describing enterovirus persistence in chronic diseases such as dilated cardiomyopathy [Bowles et al., 1986; Koide et al., 1992] and chronic fatigue syndrome (CFS) [Archard et al., 1988; Gow et al., 1991; Bowles, 1993]. CFS is a poorly understood condition, with individuals complaining of many nonspecific symptoms, but it is particularly characterised by excessive fatigue which has been present for at least six months, can be made worse by exercise and can cause significant reduction in exercise tolerance. The evidence for an association between enteroviruses (particularly Coxsackie B) and CFS has accumulated over a number of years from epidemiological data, serological studies [Bell and McCartney, 1984; Bell et al., 1988] and from nucleic acid hybridisation methods [Cunningham et al., 1990]. Investigation of Coxsackie viral infection in CFS has been based on the use of the neutralisation test (Bell and McCartney, 1984] and also the ELISA for enterovirus specific IgM [Calder et al., 1987]. However, much attention has recently been focused on the detection of viral nucleic acid as a diagnostic aid [Zoll et al., 1992]. The principal target of the polymerase chain reaction (PCR) when applied to enteroviruses has been the 5′ nontranslated region (NTR) of the genome, which is highly conserved between enteroviruses. Oligonucleotide primers to the 5′NTR are therefore enteroviral group-specific and this technique has been used to detect enteroviral RNA in muscle biopsies, stools, throat swabs and serum samples [Gow et al., 1991; Zoll et al., 1992; Clements et al., 1995]. In this study, two patient groups were examined. The study group consisted of individuals who had been diagnosed as suffering from CFS, and the comparison group consisted of "healthy" patients whose samples had been received at the laboratory for antenatal or occupational health reasons. Both groups were examined for the presence of Coxsackie B virus antibody using the neutralisation test, and for enteroviral RNA using PCR.

Patient and Comparison Groups

One hundred serum samples from CFS patients were examined. These were obtained from patients visiting

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TABLE I. Results of Coxsackie B Antibody Neutralisation Test of Study and Comparison Patients

	B1	B2	B3	B4	B5	B2 + B4	B3 + B4	Other[a]	Negative
Study	1	6	2	16	2	3	1	3	66
Comparison	2	16	1	10	7	3	0	2	59

[a]Other refers to either Coxsackie B2 + B4 + B5(2) or B1 + B4(1) in the study group, and Coxsackie B4 + B5(1) or B1 + B2(1) in the comparison group.

the outpatient Clinic of the Department of Infection and Tropical Medicine, Ruchill Hospital. For a diagnosis of chronic fatigue, the patients had to fulfil the Oxford criteria for CFS which states that there must be at least the presence of debilitating fatigue as a new symptom, persisting or relapsing for six months and that there should be the absence of disorders producing a similar fatigue type syndrome [Sharpe et al., 1991]. Specimens of venous blood were taken and transferred to the Regional Virus Laboratory where they were stored until required. The demographics of the study population were 36 males (average age 35.8 years, standard deviation 10.87, age range 17–62 years), 64 females (average age 37.0 years, standard deviation 12.75, age range 12–65 years). The duration of fatigue ranged from 6 months to eleven years (males average duration 35.4 months, females average duration 26.6 months).

One hundred serum samples from comparison patients were examined. This group consisted of individuals whose samples had been received for occupational health or antenatal screening purposes. These were obtained from the routine virological service provided at the Regional Virus Laboratory. The groups were matched by age, sex and date of specimen receipt. The age was matched to within plus or minus three years and date of specimen received to within plus or minus one month.

MATERIALS AND METHODS

Neutralisation Assay

Study and comparison sera were diluted 1/16 in phosphate-buffered saline (PBS; Sigma Ltd., Irvine, Scotland, U.K.) and heat-inactivated at 56°C for 30 minutes. Aliquots (25 μl) of each sample were transferred to 5 microtitre plates (one for each Coxsackievirus B1–B5) and double-diluted 8 times in Medium 199 (Gibco BRL, Paisley, Scotland, U.K.). Aliquots (25 μl) of the diluted Coxsackie viral stock suspensions (B1–B5) at 100 $TCID_{50}$ (tissue culture infectivity dose) were transferred into the diluted sera and the plates were incubated at room temperature for 90 minutes to allow neutralisation of the virus. After the incubation, 100 μl aliquots of Vero cell suspension (at 1.5×10^5 cells per ml) were transferred to each of the wells in the plates and incubated under CO_2 at 37°C for 3 days. Comparisons were also included for each sample to ensure that the serum alone was not toxic to the cells. The end point was taken as the dilution below that which shows cytopathic effect (i.e., the lowest dilution of serum that just neutralises the virus which has been added). In accordance with the routine laboratory method of reporting results, titres less than 256 are interpreted as not significant, titres of 256 are suggestive of enterovirus infection, and those at 512 and above are indicative of enterovirus infection.

RNA Extraction

RNA was extracted from serum (200 μl) following the methods outlined by Sambrook et al. [1989] and Chomczynski and Sacchi [1987].

Nested PCR for Enteroviral Sequence Detection

The PCR method used followed that described by Zoll et al. [1992] who established that 0.1 fg total enteroviral RNA was detected by this method (equivalent to 10 genome equivalents). Experiments were conducted blind with positive and negative controls (Coxsackie A9 infected MRC-5 cells and noninfected MRC-5 cells, respectively) to exclude false-positive and negative results.

A sample was deemed positive if a band of 264 base pairs was present after "nested" PCR and if a band was identified using the ABL primers. These sequences are present in virtually all fresh sera and we assume are derived as a result of cell degradation with release of cellular RNA. All of the controls both positive and negative must have shown the expected results in each PCR run for it to be validated. Each stage of the PCR was carried out in different dedicated microbiological safety hoods using positive displacement pipettes to minimise cross-contamination. Selected positive PCR samples were sequenced (data not shown); as each sequence was unique, this excludes the possibility of sample cross-contamination.

Data Handling

Patients previous history, present symptoms and results of laboratory tests and PCR results were stored using the data manager software package DataEase version 4.5 (1992) supplied by DataEase U.K. Ltd. Statistical analysis was performed on the data using the "odds ratio" test, (P value for alpha error = 0.05) [Gardner and Altman, 1989]. Chi-squared analysis [Daniel, 1983] was also carried out (data not shown) to corroborate the results from the odds ratio test.

RESULTS

The results for the Coxsackie B microneutralisation test for the study group and comparison group are shown in Table I. In this study, titres equal to or above

TABLE II. Comparison of Coxsackie B Antibody Neutralisation Test With Enteroviral PCR Analysis

	Enteroviral PCR (positive)	Enteroviral PCR (negative)	Total
Study group			
Coxsackie B antibody (positive)	21	13	34
Coxsackie B antibody (negative)	21	45	66
Total	42	58	100
Comparison group			
Coxsackie B antibody (positive)	4	37	41
Coxsackie B antibody (negative)	5	54	59
Total	9	91	100

256 are taken as evidence of Coxsackie B infection in the recent past and were scored as positive.

Following these criteria, 34% of the study group and 41% of the comparison group were positive for antibody to one or more of the Coxsackie B viruses. There is no significant difference at the 95% level between these findings (odds ratio = 0.74, 95% confidence interval [0.40–1.37]). In addition, statistical analysis showed that the presence or absence of any particular Coxsackie virus (B1–B5) was not associated with either study or comparison group (Table I). There was also no difference in the geometric mean of antibody titre between the study and comparison groups. In 12 individuals, there was evidence of more than one serotype of Coxsackie B virus antibody in the serum sample; this is likely to be due to cross-reactivity of the neutralising antibodies or may have in some cases been due to infection by several serotypes.

Of the 100 study group samples, 42 were positive after two rounds of PCR. The remainder did not yield a band of enteroviral product but all samples produced a band of amplified Ableson tyrosine sequence indicative of successful RNA extraction. Of the 100 comparison group samples, only 9 were positive for enterovirus PCR. Statistical analysis showed a significant difference at the 95% level between the study and comparison groups with respect to positive enterovirus PCR (odds ratio = 7.32, 95% confidence interval [4.14–17.54]).

From Table II, it can be seen that in the study group, 66 of the enterovirus PCR results were concordant with the Coxsackie neutralisation test results and in the comparison group, 58 results were concordant. There was no difference in the geometric mean of the Coxsackie B neutralisation antibody titre between the enteroviral PCR-positive and negative groups.

Of the samples that did not correlate, the majority in the study group were PCR-positive/Coxsackie B antibody-negative (21/34), whereas in the comparison group the majority were PCR-negative/Coxsackie B antibody-positive (37/42).

DISCUSSION

The investigation of serological markers to Coxsackie B virus in chronic or postviral fatigue syndrome patients has been carried out on a number of occasions. In some investigations a correlation was found; in others there was no correlation [Yousef et al., 1988; Halpin and Wessely, 1989; Miller et al., 1991]. These studies have used a variety of criteria to select CFS patients and have been carried out at different times and in different locations. It is not surprising that different conclusions have been drawn as Coxsackie Viral epidemiology varies in time and place [Minor and Bell, 1990]. The standard test for the detection of Coxsackie B virus antibody is the neutralisation test. One disadvantage of this test is that Coxsackie B virus-specific antibody levels can remain elevated for many years postinfection. IgM and IgG antibody assays on CFS patients cannot distinguish patients from the background population in some studies and a positive result does not necessarily indicate ill health [Miller et al., 1991]. The advent of PCR technology allows a different approach for the detection of enterovirus infection. Specific sequences can be identified at very low copy numbers [Gow et al., 1991; Zoll et al., 1992], and PCR-positive results from serum samples are strong evidence for a viraemia with at least one focus of replication within the body [Clements et al., 1995]. In this study we are dealing with tests which detect quite different parameters. Of the 21 patient samples in the study group that were PCR-positive but Coxsackie B antibody-negative, the results may have been due to the presence of enteroviruses other than Coxsackie B which would not have produced Coxsackie B neutralising antibody. These primers amplify the majority of enteroviruses, i.e., echo, polio, and Coxsackie A viruses as well as the Coxsackie B type [Gow et al., 1991; Zoll et al., 1992]. In fact, viral sequences other than Coxsackie B have been detected in CFS patients (Galbraith et al., submitted). In individuals who were Coxsackie B neutralising antibody-positive and PCR-negative, the lack of correlation may be due to the antibody response remaining detectable after active replication has ceased and the virus is cleared. In the comparison group, these patients have no signs of illness and therefore the Coxsackie B neutralisation test would appear to have no real clinical value for diagnosis in these groups of patients. Enteroviral PCR does, however, if positive, provide evidence for circulating viral sequences, and has been used to show that enteroviral specific sequences are present in a significantly greater proportion of CFS patients than other comparison groups [Clements et al., 1995]. Further prospective studies are in progress to

evaluate the prognostic value of enteroviral PCR testing in this condition.

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