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Detection of anti-streptococcal, anti-enolase, and anti-neural antibodies in subjects with early-onset psychiatric disorders

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Introduction. Infection with group A Streptococcus (StrepA) can cause post-infectious sequelae, including a spectrum of childhood-onset obsessive-compulsive (OCD) and tic disorders with autoimmune origin (PANDAS, Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections). Until now, no single immunological test has been designed that unequivocally diagnoses these disorders. In this study, we assessed the detection of serum antibodies against human brain enolase (AE), neural tissue (AN) and Streptococcus (AS) as a laboratory tool for the diagnosis of early-onset psychiatric disorders.

Methodology. Serum antibodies against human brain enolase, total brain proteins, and total proteins from StrepA were detected by ELISA in 37 patients with a presumptive diagnosis of PANDAS and in 12 healthy subjects from Mexico and Cuba.

Results. The antibody titers against human brain enolase (AE) and Streptococcal proteins (AS) were higher in patients than in control subjects (t-student, t_{AE}=-2.17, P=0.035; t_{AS}=-2.68, P=0.01, n=12 and 37/group, df=47, significance level 0.05), while the neural antibody titers did not differ between the two groups (P(t)=0.05). The number of subjects (titers> $mean_{control}$ + CI_{95}) with simultaneous seropositivity to all three antibodies was higher in the patient group (51.4%) than in the control group (8.3%) group (X^2=5.27, P=0.022, df=1, n=49).

Conclusions. The simultaneous detection of all three of these antibodies could provide valuable information for the etiologic diagnosis of individuals with early-onset obsessive-compulsive disorders associated with streptococcal infection and, consequently, for prescribing suitable therapy.

Keywords: Anti-neural antibodies, Auto-antibodies, Enolase, Streptococcus, Obsessive-Compulsive Disorder, PANDAS

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Detección de anticuerpos anti-estreptococos, anti-enolasa y anti-neurales en sujetos con trastornos psiquiátricos de inicio temprano

Introducción. La infección por Estreptococo del grupo A puede ocasionar secuelas post-infecciosas entre las que se han reportado un espectro de trastornos obsesivos-compulsivos y tics de aparición en la edad pediátrica y origen autoinmune (PANDAS). No ha sido diseñada una prueba inmunológica que permita diagnosticar inequívocamente estos trastornos. En este trabajo se evaluó la detección en suero de anticuerpos contra Enolasa cerebral humana (AE), tejido neural (AN) y Estreptococo (AS) como herramienta de laboratorio para el diagnostico de trastornos psiquiátricos de inicio temprano.

Metodología. Los anticuerpos séricos contra Enolasa cerebral humana, proteínas totales del Estreptococo y proteínas totales cerebrales fueron detectados mediante la metodología de ELISA en 37 individuos con diagnóstico presuntivo de PANDAS y en 12 sujetos sanos de México y Cuba.

Resultados. La títulos de anticuerpos contra AE y AS fueron más elevados en el grupo de pacientes *vs* controles (t-student, t_{AE}=-2.17, P=0.035; t_{AS}=-2.68, P=0.01, n=12 y 37/grupo, gl=47, nivel de significación de 0.05), mientras

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que los títulos de anticuerpos AN no difirieron entre ambos grupos (P(t)=0.05). La seropositividad (títulos > media$_{control}$ + IC$_{95}$) simultánea a los tres anticuerpos fue mayor (51.4 %) en los individuos del grupo de los pacientes comparado con los controles (8.3%) (X^2=5.27, P=0.022, gl=1, n=49).

Conclusiones. La detección simultánea de los tres anticuerpos séricos podría brindar información útil para el diagnóstico etiológico de los individuos con trastorno obsesivo-compulsivo de inicio temprano asociados con la infección por Estreptococo y en consecuencia para indicar una terapéutica adecuada.

Palabras Clave: Anticuerpos anti-neurales, Auto-anticuerpos, Enolasa, Estreptococo, Trastorno obsesivo-compulsivo, PANDAS

INTRODUCTION

Streptococcus pyogenes group A (StrepA) is a Gram-positive bacterium that infects human skin and mucus membranes. This microorganism is capable of causing diseases ranging from pharyngitis to cardiac dysfunction and even generalized sepsis.[1] StrepA infections can cause non-suppurative sequelae affecting different organs and systems, including the central nervous system (CNS). Among them are glomerulonephritis, rheumatic fever, and Sydenham's chorea.[2,3]

Swedo et al.[4] in 1998 proposed the general concept of pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) to denote a subtype of disorder in children characterized by tics and/or obsessive compulsive disorder (OCD) in the context of a previous streptococcal infection. The same authors evaluated these children longitudinally, in which they proposed the characteristics that define the subgroup: presence of tics or OCD symptoms of abrupt, prepubertal onset, episodic course, motor disorders, and temporal association of psychiatric symptoms with strep throat.[4-6] Given this background, it has been postulated that Sydenham's chorea (SC), obsessive compulsive disorder (OCD), and Tourette syndrome (TS) are neuroimmunologic disorders associated with the presence of antibodies originated in response to a StrepA infection that have cross reactions with brain tissue.[7-10]

In recent years, evidence has been found of autoimmune mechanisms that may be directly involved in the pathophysiology of certain OCD subtypes, such as PANDAS.[4,5] Although StrepA bacteria cause between 15% and 30% of cases of acute pharyngitis in pediatric ages,[11,12] only a small percentage of those affected develop post-streptococcal autoimmune diseases. This implies that these diseases develop only in a susceptible host infected by a specific StrepA serotype.

The first laboratory tests used to demonstrate the autoimmune origin of neuropsychiatric post-streptococcal sequelae were based on determining the simultaneous presence in serum of streptococcal antibodies against the M protein in cell membranes and of anti-neural antibodies (AN).[13,14,16] The presence of these antibodies showed that the subjects had prior exposure to Streptococcus group A. These antibodies persist for several years after the streptococcal infection occurs.[14-16] Dale et al.[14,17] identified antigens present in the brains of humans and rats to which AN antibodies present in individuals with post-StrepA infection neuropsychiatric diseases bind. These antigens had molecular weights of 40, 45, and 60 kDa and were identified as pyruvate kinase, aldolase C, and enolase.

The timing between StrepA infection and the onset of tics or OCD symptoms makes it difficult to identify the molecules involved in the mechanism of production of the disease. It is thus necessary to know the factors involved in the onset of tic and/or obsessive-compulsive symptoms in children after strep infection to improve diagnosis and/or treatment of these conditions and contribute to the potential development of vaccines to prevent them.

In this study we determined the serum titers of antibodies against Streptococcus (AS), total brain tissue (AN) and the neuronal enzyme enolase (AE) in children from Cuba and Mexico with early-onset psychiatric disorders in order to assess possible tests as laboratory tools for diagnosing PANDAS.

METHODOLOGY

We randomly selected 37 patients diagnosed with abrupt onset OCD and/or tics and 12 neurologically and psychiatrically normal subjects under 18 who had been treated at the Adolescent Clinic in Havana or the Carracci Medical Group Clinic in Mexico City. The participating subjects, or their legal representatives in the case of minors, signed an assent and informed consent, which was previously approved by the ethics committee of the health institutions involved.

Rheumatic fever, Sydenham's chorea, or autoimmune disorders in the history was considered an exclusion criterion.

The presence of obsessive-compulsive disorder and/or tics was verified using the criteria of the Diagnostic and Statistical Manual of Mental Disorders[18] (DSM-IV), based on the MINI (Modified International Neuropsychiatric Interview) psychiatric interview and the International Classification of

Diseases[19] (ICD-10), the abrupt, early onset of symptoms and episodic course (acute exacerbations, independent of temporal course), and a history of recurrent infections. Comorbidity and the severity of the obsessive-compulsive disorder were evaluated using the scales and diagnostic interviews approved by the International College of Obsessive Compulsive Spectrum Disorders (ICOCS), which include questionnaires addressing the obsessive compulsive spectrum and motor tics. These questionnaires were previously validated in Spanish.[20,21] Once enrolled, patients were administered structured and semi-structured scales for psychological evaluation.

It was also verified that the control group subjects had normal results in a clinical neurological and psychiatric examination at enrollment, and did not have a history of recurrent infections, or neurological or psychiatric diseases.

The composition of the sample by age group and sex is shown in Table 1. The age of that participants was between 2 and 17 years, with a mean (SD) of 11.5 (4.3) years. There were no differences age between the individuals in the control group and patients (P=0.05). However, in the group of patients the frequency of male individuals was higher than in the control group (Yates(X^2)=6.23, df=1, P=0.013) (Table 1).

A blood sample was drawn from all the individuals participating in the study for the determination in serum of antistreptolysin-O titers and the presence of antibodies against neural tissue, neuronal enolase, and Streptococcus total protein.

Serum antistreptolysin-O titers were detected using a latex agglutination kit for which the manufacturer's specifications (Centis Diagnósticos® ASO-Latex, Havana, Cuba) were followed. Antibodies against streptococcus, cerebral enolase, and neuronal tissue were detected in serum using indirect ELISA methodology, and the antigens used were total extracts of StrepA, adult Wistar rat brain, and human neural enolase protein.

Total streptococcus extracts (StrepA) were obtained from the *Streptococcus pyogenes* Group A serotype M3 strain, ATCC 12384. Bacteria were cultured at 37°C overnight and harvested by centrifuging. Sonicated bacterial cells were suspended in phosphate buffer 0.1M, pH 7.4 (1X PBS) and stored at -70°C until use.

Anti-neural brain antigens were extracted from the brain of healthy adult male Wistar rats by homogenizing the tissue, previously frozen at -70ºC, in T-PER Tissue Protein Extraction Reagent (Pierce, USA).

The proteins present in total extracts of StrepA and rat brain were quantified by the Lowry method and their integrity and molecular weight was verified by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

The neuronal enzyme enolase (Neuron-specific Enolase from Human Brain, Sigma N4773) was diluted according to the manufacturer's instructions.

Tests were performed using ELISA methodology by diluting each antigen in sodium carbonate/bicarbonate buffer solution 0.05M (pH 9.6) at a concentration of µg/mL. We added 100 µL of this solution to the wells of an ELISA plate and left it to incubate overnight at 4°C. The wells were then washed 4 times with 0.05% Tween. The wells coated with each of the antigens were incubated with 5% skim milk in 1X PBS for 1 hour at 37ºC, and the plate was again washed 4 times with 0.05% Tween (v/v). We added 100 µL of patient serum diluted in 1X PBS plus 0.05% Tween to each well and left the plate to incubate 1 hour at room temperature.

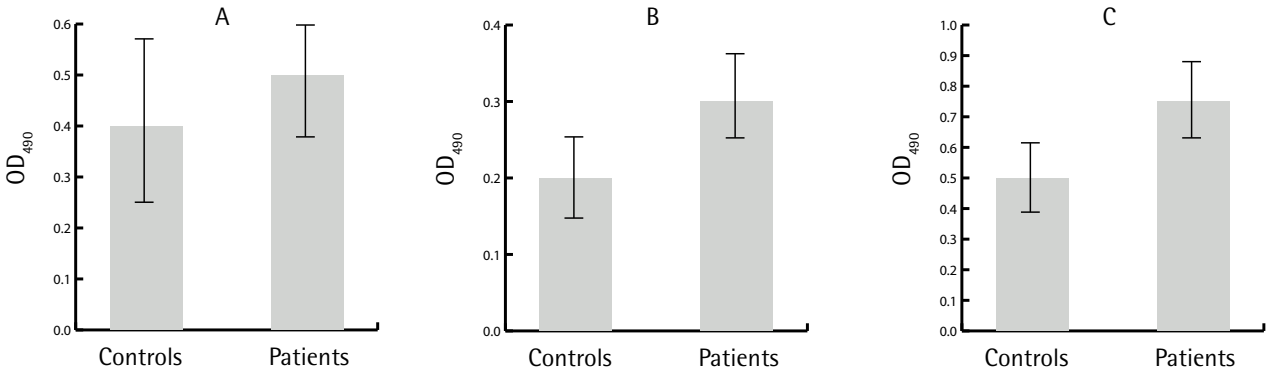
Subsequently, the wells were washed 4 times with 0.05% Tween (v/v); 100 µL of peroxidase-conjugated anti-human immunoglobulin G (Sigma-Aldrich) diluted (1/10000) with 1% skim milk in 1X PBS plus 0.05% Tween was added to each, and the plates were incubated 1 hour at 37ºC. The plate was washed 4 times with 0.05% Tween (v/v), and 100 µL of O-phenylenediamine (OPD) 0.1% was finally added to each well as developer solution, and the reaction was allowed to take place in the dark for up to 15 minutes. The reaction was stopped by adding 50 µL of H2SO4 2.5M. The absorbance values were read at a wavelength of 490 nm. Each test was performed at least in triplicate for each study participant.

The results were analyzed using the statistical package StatSoft, Inc. (2007) STATISTICA (data analysis software system Version 8.0. www.statsoft.com). The level of significance was 0.05. Continuous variables were compared with the Student t-test for independent samples or the

Table 1 Demographic variables of the individuals screened

		Controls	Patients	P value
Individuals (n)		12	37	
Age (m+SD)		9.5 ± 5.4	12.2 ± 3.8	0.064*
Gender n (%)	Female	7 (58.3)	6 (16.2)	0.013**
	Male	5 (41.7)	31 (83.8)	

m: mean; SD: standard deviation; * T probability of t in the Student-t test for independent samples at a significance level (α) of 0.05; ** denotes significant differences between groups in the Chi-square test (α=0.05).



Results are expressed as mean ± CI95 of the optical density at 490 nm (OD_{490}) measured in ELISA. The homocedasticity of the measurements was verified with the Brown-Forsythe test ($\alpha = 0.05$).

Figure 1 | *Titers of anti-neural (A), anti-enolase (B), and anti-streptococcal antibody (C) in patients and controls*

Hotelling T2, while dichotomous variables were compared with the Chi-square test. The homocedasticity of the data was evaluated by the Brown-Forsythe test.

In order to consider the individuals of the patient group as "positive" in each of the tests performed, the ELISA upper cutoff value of was calculated as the mean of the absorbance values obtained in the control group was calculated for each of the antigens detected, plus the 95% confidence interval of the mean (mean + CI_{95}). The "positive" individuals 'in the control group were identified for each test group by evaluating using the statistical technique called "leave one out." This was done by excluding one individual in the control group, calculating the mean and CI_{95} for the group, and verifying whether the determinations for an individual were within mean ± CI_{95} interval. The individual was then included in the group and the next individual was excluded; the above procedure was repeated successively until each of the individuals in the control group was evaluated.

RESULTS

At the time of recruitment, although all the subjects in the control group were healthy and did not report streptococcal infections, 25% of them has antistreptolysin-O titers of 200 IU/mL. The presence of obsessive-compulsive disorder was verified in all patients, but only 50% of them had motor and/or vocal tics. The number of male individuals affected exceeded that of females (5.2:1), and the gender composition of the control and patient groups was different (Table 1). All the patients reported a history of recurrent infections, including streptococcal infections, and at recruitment 21.6% of them presented antistreptolysin titers of 200 IU/mL while 16.2% had titers higher than 400 IU/mL.

The titers of anti-enolase (AE) and anti-streptococcal (AS) antibodies were higher in patients than in controls (t-student, t_{EA}=-2.17, P=0.035; t_{SA}=-2.68, *P*=0.01, n=12 and 37/group, *df*=47, significance level 0.05), whereas no differences between groups were observed in the titers of anti-neural (AN) antibodies (*P*(t)=0.05) (Figure 1). However, the analysis of the profile of the antibody titer set against all three antigens yielded differences between patients and controls (Hotelling T^2=9.75, F(3,45)=3.11, *P*<0.036).

The percentage of individuals in the group of patients with positive titers (titers>mean of the controls + CI_{95}) in the test for AE antibodies was higher (86.5%) than that of the control group (16.7%) (Yates X^2=17.64, *P*=0.00003, *df*=1, n=49) (Figure 2).

Similarly, a higher percentage of positive results was observed in patients (86.5%) than in controls (33.3%) in AS antibody screening (Yates X^2=10.55, *P*=0.001, *df*=1, n=49) (Figure 2).

No differences were detected between the frequency of positive results in patients and controls for serum AN antibody screening (*P*(Yates X^2)=0.05) (Figure 2).

In the overall analysis of positive test results for AN, AS, and AE antibody screening, 94.6% of individuals in the group of patients had positive results for at least one antibody, compared with 33.3% of individuals in the control group (Yates X^2=17.33, *P*=0.00003, *df*=1, n=49). Consequently, the frequency of individuals with simultaneously negative results for the three antibodies evaluated was sig-

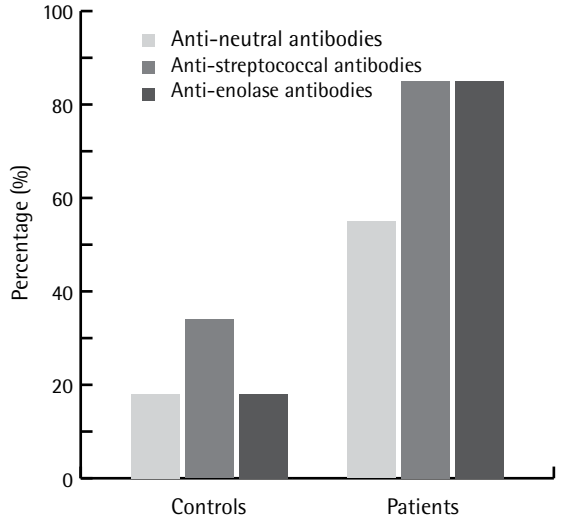


Figure 2 — *Frequency of seropositive results in ELISA for the detection of anti-neural (AN), anti-streptococcal (AS), or anti-enolase (AE) antibodies (Ab)*

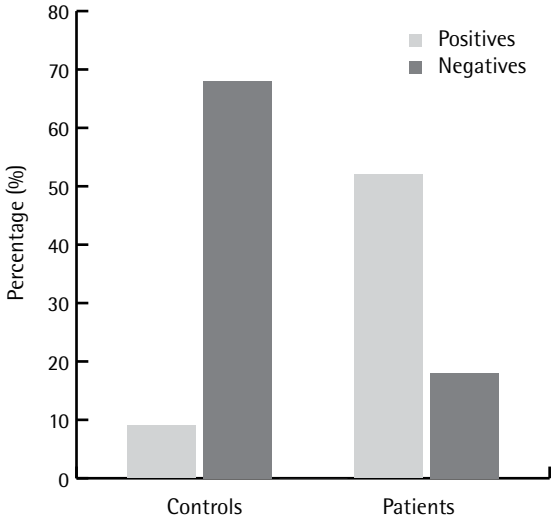


Figure 3 — *Frequency of simultaneous seropositivity or seronegativity in ELISA for screening of anti-neural (AN), anti-streptococcal (AS), and anti-enolase (AE) antibodies*

nificantly higher in the control group than in the patient group (P (Yates X^2)≤0.006). However, the percentage of individuals in the patient group who had simultaneously positive results in all three tests (51.4%) was higher than in the control group (8.3%) (Yates X^2=5.27, P=0.022, df=1, n=49) (Figure 3).

The profile of antibody titers against all three antigens was different between patients and controls (P(Hotelling T^2)<0.036). The frequency of patients with simultaneously positive results for all three antibodies (AN, AS, and AE) was significantly greater among patients than controls ($P(X^2)$≤0.05, Figure 3), so this group of patients with positive results could be labeled as PANDAS whereas the individuals who did not have any of the three antibodies could be regarded as true controls.

CONCLUSIONS

Notwithstanding that recurrent exposure to *Streptococcus pyogenes* occurs in all individuals at the pediatric age,[11,12,22] a greater proportion of patients than controls had AS antibodies (Figure 2). This result suggests that StrepA could be involved in the pathogenesis of obsessive-compulsive disorder (OCD), as has been reported by other authors.[23] Moreover, the presence of auto-antibodies against enolase in a greater percentage of patients than in controls (Figure 2) is consistent with reports from other authors regarding this finding in individuals with OCD and previous recurrent StrepA infections.[7-9] In this study, a higher frequency of male than female individuals with obsessive-compulsive disorder beginning in childhood and a history of recurrent infections was found (Table 1), which is consistent with previous reports in the literature and suggests the homogeneity of the diagnostic criteria for PANDAS used in the study.[5]

The lack of differences between the serum titers of AN antibody could be due to the high variability of the results of these tests ($SD_{control}$=0.248 and $SD_{patients}$=0.272), or the use of rat neural tissues, although it has been reported in the literature that they behave as heteroantigens in patients with PANDAS.[14,17]

The low percentage of true controls (participants in the control group without titers of antibodies against AN, AS, and AE antigens) could be due to repeated exposure to StrepA strains during life, particularly in childhood.

The determination of serum titers of AS, AN, and AE antibodies could be useful for the early diagnosis of patients with PANDAS and thus contribute to initiating adequate therapy. Although the proposed tests are not specific for the diagnosis of PANDAS, these results provide evidence of the possible autoimmune origin of this disorder and should contribute to the search for biomarkers that may be involved in its pathogenesis.

Determining the simultaneous presence of all three antibodies in individuals with OCD may facilitate early diagnosis of PANDAS and providing a specific and not just symptomatic treatment from the onset of the disease. It would be appropriate to implement the detection of other antigens specific to brain tissue and StrepA, which would increase the sensitivity and specificity of these tests.

LIMITATIONS AND FUTURE PROSPECTS OF THE STUDY

One limitation of this study is the small number of participants who were recruited. Consequently, the participants might not be representative of the general population. This investigation is also limited by its cross-sectional design. It is necessary to carry out a larger confirmatory study and longitudinal follow-up of children with recurrent infections who have not yet developed obsessive-compulsive disorder to better assess the association between the presence of autoantibodies and such disorders and evaluate the possible predictive value of these tests and their value in the diagnosis of PANDAS. It is also advisable to increase the sensitivity and specificity of the proposed tests for detecting autoantibodies by previously identifying other molecules involved in the disorder.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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