

## ENZYME LINKED IMMUNOSORBENT ASSAY: DETERMINATION OF ANTI-ADENOVIRUS ANTIBODIES IN AN INFANT POPULATION

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### SUMMARY

In order to define an accurate assay for anti-adenovirus antibody detection, a recently developed ELISA was compared with IFA and CF. On 58 sera, the ELISA was more sensitive than both CF and IFA, which showed relative sensitivities of 63% and 94%, respectively. It was not possible to determine the exact specificity of the tests because of the lack of a gold standard. Furthermore, the ELISA was used to define the prevalence of adenovirus antibodies in 116 infants between 1 and 24 months old (mean 7.28). The data showed that maternal antibodies waned by the age of 5 to 6 months and that more than 80% of the children had been infected by adenoviruses by the age of 10 months.

**KEY WORDS:** Adenovirus serology; Enzyme linked immunosorbent assay; Adenovirus infection in children.

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### INTRODUCTION

Adenoviruses (Ad) are ubiquitous human pathogens that commonly produce benign respiratory, gastrointestinal, urinary and ocular diseases. However, adenovirus infections may be extremely severe and often lethal in immunocompromised hosts such as transplant recipients, cancer patients undergoing chemotherapy, children with severe combined immunodeficiency and AIDS patients<sup>2, 4, 9, 11, 16</sup>. Previous studies in transplant patients have suggested that the most likely source of infection was endogenous reactivation of latent virus<sup>12</sup>. In this population routine surveys are very important to identify the individuals at risk of developing adenovirus disease.

Laboratory diagnosis of adenovirus infections has relied on recognition of cytopathic effect in tissue culture, monoclonal antibodies mediated rapid detection<sup>5, 6</sup> or significant increases of antibody titers in paired sera. Anti-Ad IgM antibody assays have not yet been standardized. With the recent advances in antiviral therapy, sensitive diagnostic methods are needed to disclose the infectious agents early in the course of the disease and to determine the candidates to antiviral prophylaxis<sup>10</sup>.

The goal of the present study was to test the diagnostic accuracy of an IgG anti-Ad ELISA developed in our laboratory. We also exami-

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ned the prevalence of adenovirus infection in young children in order to complete previous epidemiologic surveys and establish background information of adenovirus risk of infection for pediatric transplant patients in our environment.

## MATERIAL AND METHODS

**Viruses and cells.** KB, Hep 2 and human foreskin fibroblast (HFF) tissue cultures were maintained at 37°C in Eagle's minimum essential medium (MEM) (Gibco, Grand Island, NY) complemented with 10% fetal calf serum (FCS) (Cultilab, Campinas, SP) and 100 U/ml penicillin, 100 µl/ml streptomycin and 2 µl/ml amphotericin B. Ad 5 was propagated on Hep 2 and KB cells in MEM containing 2% FCS and antibiotics. Infectivity was measured by TCID<sub>50</sub> in KB tissue culture tubes or by plaque formation on HFF as previously described<sup>15</sup>. Titers varied between 10<sup>5</sup> plaque forming units/ml and 10<sup>7</sup> TCID<sub>50</sub> ones.

**Serum samples.** 116 blood samples were obtained from children visiting the vaccination centers of the Hospital do Servidor Público Estadual and Hospital das Clínicas da FMUSP. The sera were stored at -20°C until tested. Another 58 serum samples from patients referred to the Instituto Adolfo Lutz for clinically suspected adenovirus infection were used for the comparison of the anti-Ad antibody detection methods.

### Antibody detection.

The complement fixation (CF) technique used for anti-Ad antibody determinations was already published<sup>13</sup>.

**ELISA for anti-Ad antibodies** was adapted from a previous publication<sup>14</sup>. Briefly, the antigen was prepared from 3 day old Ad 5 infected KB monolayers at complete CPE and mock infected KB tissue cultures. Cells were washed in phosphate buffered saline (PBS), pH 7.6, and resuspended in 0.1 M glycine in PBS at a volume 10 times greater than the packed cell volume. The suspension was sonicated, solubilized with 1% Tween 80 (Merck, Rio de Janeiro, RJ) and clarified by centrifugation. 96 well microtiter plates (Hemobag, Campinas, SP) were coated with viral and mock antigen and the suspensions we-

re allowed to evaporate overnight at 37°C. The optimal antigen dilution was found by checkerboard titration using a serum of known anti-Ad antibody titer. The plates were sealed in plastic bags and stored at -20°C until used. There was no detectable loss in antigenicity after 4 months of storage. Before use, the plates were washed with PBS containing 0.5% Tween 80 (TPBS) and uncoated sites were blocked with 1% bovine serum albumin (BSA) in TPBS. Duplicates of 1/20 dilutions of the test sera in TPBS containing 1% BSA were added to the viral and mock coated wells and incubated for 30 min at 37°C. Then, the plates were washed and incubated with goat anti-human IgG antibodies conjugated to horseradish peroxidase (Sigma, St. Louis, Mo). The reaction was revealed with o-phenylenediamine (Sigma) and read with a Titertek Multiskan MK II (Flow, McLean, Vi). Specific binding was considered to occur when the difference between the mean absorbance in viral and mock antigen wells was higher than 0.1.

**Indirect immunofluorescence assay.** Two day old Ad 5 infected Hep 2 monolayers and uninfected controls were washed, resuspended in PBS and acetone fixed onto slides at a concentration of 30 to 50 cells per 400 x high power field. Thereafter, 25 µl of 1/10 diluted serum samples were added to the slides and incubated for 30 min at 37°C in a wet chamber. Then, the slides were washed with PBS and incubated with anti-human IgG fluorescent conjugate (Sigma) for 30 min at 35°C. After another 5 min incubation with Evans' blue, the slides were washed, mounted in 90% glycerine in PBS and read with a 50 W Nikon epifluorescent microscope.

## RESULTS

### Comparative analysis of ELISA, CF and IFA for anti-Ad antibodies detection.

In order to determine the diagnostic power of the newly developed anti-Ad ELISA, 58 sera obtained from patients with clinically suspected adenovirus infection were tested for specific antibodies using ELISA, as well as previously established IFA and CF. Table 1 shows that the ELISA test displayed the highest sensitivity, 57 positive results, followed by IFA, 54 seropositives (94% sensitivity compared with ELISA) and at

TABLE 1  
Comparative anti-Ad antibody detection by ELISA, IFA and CF in 58 sera.

	Seropositives	Seronegatives
ELISA	57	1
IFA	54	4
CF	36	22

last the CF (36 seropositives) with a sensitivity of 63% compared with ELISA and 66% compared with IFA. All the samples seropositive by CF were also positive by ELISA. In contrast, one of the CF and ELISA positive sera was negative in the IFA assay, indicating the lower sensitivity of IFA compared to ELISA. The serum that was negative for anti-Ad antibodies by ELISA resulted also negative with the use of the other two tests. Two sera were positive by ELISA and negative according to both CF and IFA assays.

#### Anti-Ad antibody prevalence in infants from the city of São Paulo

116 serum samples obtained from children between 1 and 24 months of age, mean of 7.28 months, were tested for the presence of anti-Ad antibodies using ELISA, as shown in Fig. 1. The data indicate that infants of less than 4 months of age have a high prevalence, close to 100% of anti-Ad antibodies, most of them probably acquired intrauterus. At the age of 5 to 6 months the curve shows a nadir, suggesting the loss of

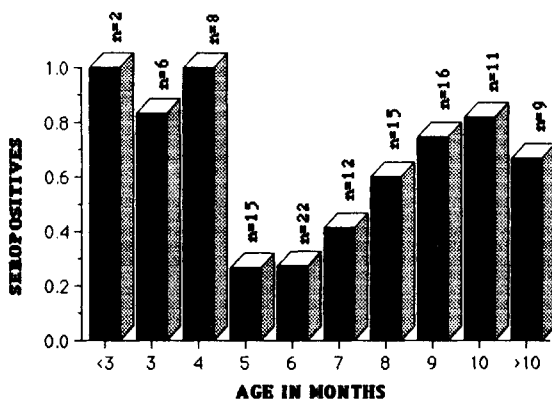


Fig. 1 — The data were derived from 116 sera tested by ELISA for anti-Ad antibodies presence. n = number of sera in each group.

maternal antibodies. However, after 7 months of age the anti-Ad antibody level increases again reaching 85% positivity at 10 months. This last raise represents newly acquired anti-Ad antibodies as a consequence of Ad infection in the study population.

#### DISCUSSION

In this study, we showed that the ELISA was more sensitive for anti-Ad antibodies detection than CF and IFA, which displayed relative sensitivities of 63% and 94%, respectively. Our results are in accordance with data obtained in other studies<sup>7</sup>, where the ELISA had both higher sensitivity and specificity when compared with IFA or CF. However, in our system, it was difficult to assess the specificity of the ELISA, since the two sera which were antibody positive by ELISA and negative by CF and IFA could represent either false positive ELISA results, or false negative CF and IFA ones. These differences could not be ascribed to the specificity of the antigens employed in the three assays, since all of them used the same Ad 5 strain for viral antigen preparation. The Ad 5 has been previously shown to extensively cross react with antibodies to the other known Ad strains, by CF and ELISA<sup>7</sup>. However, this observation does not hold true for anti-Ad neutralizing antibodies, which are highly type specific. Therefore, although the neutralization reaction represents the gold standard for anti-Ad antibodies determination, it could not be used in this study, because we did not know which strains had infected the patients whose sera were tested. The accuracy of IFA was very close to the ELISA one in this study, but the ELISA offers additional advantages, since it is an uncomplicated test to perform and, in contrast to IFA, it is automatically read and does not depend on the visual skills of the lab technologist.

We also found a high prevalence of anti-Ad antibodies in the infants from the city of São Paulo. The antibody curve showed a sudden fall from more than 95% seropositives before 4 months of age, to 27% positive samples at 5 and 6 months of age. The nadir most probably represented the loss of maternal antibodies. Thereafter, there was a steady increase in the prevalence of anti-Ad antibodies, indicating true adenovirus infec-

tions in this population, so that after 9 months of age more than 80% of the children had been in contact with these viruses. These findings are in accordance with previous studies<sup>1, 13</sup> which demonstrated that although adenoviruses accounted for 5.4% of the respiratory infections in infants between 0 and 24 months of age, in São Paulo, these viruses could not be isolated from children younger than 4 months. Moreover, it has already been shown that, in the city of São Paulo, after the age of 2 years 76.6% of the population had anti-Ad antibodies by CF<sup>3</sup>. The data presented in our study support the conclusion that most of the primary adenovirus infections are already acquired by the age of 10 months. However, the presence of anti-Ad antibodies does not necessarily mean protection against disease, because neutralizing defenses are very much type specific and reinfections with different serotypes are quite frequent. Furthermore, the adenoviruses may become latent and in the immunocompromised hosts the reactivations, although less frequent than with herpesviruses, can carry an equally high morbidity. In view of the present expansion of immunosuppressive therapeutic regimens, routine surveys of adenovirus reactivations or reinfections are needed, beginning at young ages and particularly in bone marrow and liver transplant patients which appear to bear the highest risk of fatal adenovirus infection<sup>2, 10, 11</sup>.

In conclusion, our data have demonstrated that ELISA is a highly sensitive test for anti-Ad antibodies detection, superior to CF and IFA. We have also shown that adenoviruses start to spread, in São Paulo, at very early ages and that more than 80% of the children are at risk of reactivation upon immunosuppression.

## RESUMO

### Ensaio imunoenzimático: Detecção de anticorpos anti-adenovirus numa população infantil de São Paulo.

Com a finalidade de encontrar um ensaio preciso para a detecção de anticorpos anti-adenovirus, o teste ELISA recentemente padronizado foi comparado à imunofluorescência indireta (IFI) e à fixação de complemento (FC). Após testar 58 soros, o ELISA demonstrou maior sen-

sibilidade do que a IFI e a FC, que mostraram sensibilidades relativas de 94% e 63%, respectivamente. A falta de um padrão universal não permitiu alcançar conclusões definitivas quanto à especificidade dos ensaios. Além disso, o ELISA foi utilizado para estabelecer a prevalência de anticorpos anti-adenovirus em 116 crianças entre 1 e 24 meses de idade (média 7,28). Os dados mostraram que os anticorpos maternos desaparecem ao redor dos 5 a 6 meses de idade e que mais de 80% das crianças tinham sido infectadas antes dos 10 meses de idade.

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