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A collaborative study on larval excretory/secretory antigens of *Toxocara canis* for the immunodiagnosis of human toxocariasis with ELISA*

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Summary

Two batches of excretory/secretory (E/S) antigens from second stage larvae of *Toxocara canis* maintained in vitro were prepared independently in two different laboratories (Zürich and Basel) and analysed in order to obtain information for future efforts to standardize the enzyme-linked immunosorbent assay (ELISA) used for the serodiagnosis of human toxocariasis. SDS-PAGE and "Western-blotting" revealed at least 10 different antigenic components common to the two antigen preparations. However, distinct qualitative and quantitative differences among the two E/S-antigens were observed, since one antigen had a more complex composition than the other. Despite these differences, an accordance of serodiagnosis was obtained in 80% of 25 sera from patients with suspected *Toxocara* infection tested independently in two different ELISA systems (Basel and Zürich) with the corresponding E/S-antigens. The specificity was 93% as determined (BS-antigen, BS-ELISA) by testing 46 out of 3396 sera from patients with parasitologically proven extra-intestinal helminthic infections. Cross-reactions occurred mainly with sera from patients infected with filariae (5 from 13 cases) exhibiting very high extinction values in their homologous ELISA-system. The reproducibility (intra- and inter-test variations) of two ELISA systems using the corresponding E/S-antigens varied from 5-15%. The results demonstrate that *T. canis* E/S-antigens may well be applicable for standardization of the ELISA used for the serodiagnosis of human toxocariasis.

Key words: *Toxocara canis;* immunodiagnosis; standardization; SDS-PAGE; Western-blotting.

* In cooperation with the "Arbeitsgemeinschaft Immundiagnostik" and participation of Proff. M. Stoye, J. Lamina and J. Eckert

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Introduction

Excretory/secretory (ES) antigens derived from second stage larvae of *Toxocara canis* maintained in a defined medium in vitro (de Savigny, 1975) have been used by various authors for the serodiagnosis of human toxocariasis in the enzyme-linked immunosorbent assay (ELISA) (de Savigny et al., 1979; Carlier et al., 1982; Matsumura and Endo, 1982; Yang, 1982; van Knapen et al., 1983) and latex agglutination test (Sugane and Oshima, 1983). In these studies the ELISA test has been shown to be as sensitive as a radioimmunoassay with *T. canis* E/S-antigens (de Savigny and Voller, 1980). The ELISA also exhibits a high degree of specificity (de Savigny et al., 1979; Van Knapen et al., 1983).

Epidemiological studies carried out in various countries revealed that antibodies against *T. canis* were detectable in the sera of 2–11% of the persons examined (de Savigny et al., 1979; Carlier et al., 1982; Matsumura and Endo, 1982; Van Knapen et al., 1983). The wide variability in seroreactivity may well reflect different epidemiological situations and incidences of *Toxocara* infections in humans. However, an exact comparison of data from different areas and laboratories is unfeasible as long as the serological techniques are not standardized.

Standardization of serological procedures for the diagnosis of parasitoses in humans is an urgent requirement (Kagan, 1982). The main obstacle to achieve this aim is the lack of defined antigens (Eckert and Gottstein, 1983).

The intention of the present work was to contribute to standardization by comparing the quality of two batches of *T. canis* E/S-antigens in the ELISA. The antigens had been isolated according to the same principles (de Savigny, 1975) in two different laboratories in Basel and Zürich, Switzerland.

Materials and Methods

Experimental design

1. Two different batches of *T. canis* E/S antigens were independently prepared in two different laboratories: one in the Swiss Tropical Institute in Basel (BS-antigen) and one in the Department of Parasitology at the University of Zürich (ZH-antigen).
2. The BS- and ZH-antigens were comparatively analysed with SDS-PAGE and the "Western-blot" techniques.
3. Both antigen preparations were simultaneously tested in the same ELISA system with different serum groups in order to analyse the distribution of extinction values.
4. Both antigen preparations were tested by two independent ELISA systems with sera from patients with suspected *Toxocara* infection.
5. The data obtained during a one year routine application of the BS-antigen in the ELISA system in Basel were analysed with regard to cross-reactions.
6. The reproducibilities of the two ELISA systems using the two batches of E/S antigens were evaluated using the same positive and negative serum control pools.

Antigens: Eggs of *T. canis* were isolated and incubated in both laboratories independently according to the method of Annen et al. (1975). The in vitro cultivation of the second stage larvae and the collection of the two different batches of E/S-antigens was carried out according to de Savigny (1975). The larvae were maintained at a concentration of about 6000/ml in Falcon culture

bottles (type 3012) in MEM medium (Zürich: Serva, Heidelberg, Nr. 47365 A; Basel: Inotech Nr. LM-159-1) which contained 100 U penicillin and 250 μg streptomycin per ml (ZH) or 200 U penicillin and 200 μg streptomycin per ml (BS) respectively. The medium was changed once a week. Cultures with more than 7% deadlarvae were discarded in both laboratories. The culture supernatants were filtered through a 0.22 μm Milliporefilter, dialyzed against distilled water and stored at $-20°$ C until further processing (collection time in Basel and Zürich: 3 months). The collected supernatants were then concentrated in an Amicon ultrafiltration cell (YM-10 membrane in Zürich, PM-10 membrane in Basel) or Millex CX 10 (Basel). The BS-antigen was ultracentrifuged (100000 *g*, 4° C, 2 h), but the ZH-antigen was used without ultracentrifugation. The protein amounts of the final antigen solutions were determined according to the Lowry method or with the Biorad Protein Assay. No difference was found between the two methods. All antigens were kept frozen at $-70°$ C in small aliquots until used for sensitizing ELISA plates.

SDS-PAGE: All SDS-PAGE chemicals, including molecular weight markers, were obtained from BIO-RAD Laboratories. Gels were cast and electrophoresed in a LKB 2001 vertical electrophoresis unit. SDS-PAGE was performed according to Lämmli (1970) using a 4% stacking and a 10% running polyacrylamide gel. 80 μg (prepared by lyophilization) of *T. canis* E/S-antigens of both laboratories were dissolved in 30 μl of a solution containing 1% SDS, 5% mercaptoethanol, 10% glycerol, 10 mM Tris base and 1 mM EDTA. The samples were heated to 100° C for 3 min prior to electrophoresis. The protein staining was achieved by the BIO-RAD silver stain.

"Western-blot": Antigenically active components among SDS-PAGE resolved bands were detected by the "Western-blotting" method as described by Gottstein (1983). After SDS-PAGE, the proteins were transferred electrophoretically onto a nitrocellulose sheet (BA 85 Schleicher and Schüll), using an Electro-blot TM system from E-C Apparatus (UniEquip, München, BRD). In order to block remaining absorptive sites of the nitrocellulose sheet an incubation of 30 min was then performed in a 50 mM Tris-HCl buffer (pH = 7.4) containing 0.5% BSA, 0.25% gelatine (Fluka, Buchs, Switzerland), 0.05% Nonidet NP40 and 140 mM EDTA. The same buffer solution was used for diluting the positive and negative human serum pools (1:200) as well as for the second sandwich-antibodies (swine anti-human IgG (Fc) (Orion Diagnostica Nr. D-743) labelled with [^{14}C]formaldehyde (Amersham SW Ldt UK) by the method of Jentoft and Dearborn (1979). The radioactive bands were visualized by fluorography, using a surface autoradiography enhancer spray (NEN, NEF-970 and Kodak XAR-2 film). Exposure took place at $-80°$ C for 12 hours.

The positive and negative human serum pools were prepared from 30 reference sera of patients with suspected visceral larva migrans and of healty blood donors, respectively.

Serum samples

a) 20 frozen sera from the Children's Hospital in Berne (Switzerland). The children (5–15 months old) had no history suggesting toxocariasis and were hospitalized mainly in relation to accidents.
b) 47 frozen sera from normal blood donors from a village in the southern part of Jura (Switzerland).
c) 25 lyophilized sera from Swiss patients with suspected *Toxocara* infection.
d) 3396 fresh sera from the routine diagnostic work of 1982. Among these sera 46 were from proven cases of the following parasitoses: 15 schistosomiasis (12 *Schistosoma mansoni*, 2 *S. haematobium*, 1 *S. japonicum*); 13 filariasis (8 *Dipetalonema perstans*, 4 *Onchocerca volvulus*, 1 *Loa loa*); 16 echinococcosis (13 *Echinococcus granulosus*, 3 *E. multilocularis*); 2 sera came from patients with fascioliasis (*Fasciola hepatica*).
e) The positive control serum was a pool consisting of 30 different sera from patients with a clinical history suggesting toxocariasis and reacting with *T. canis* E/S-antigen in ELISA.
f) The negative control serum was a pool of 30 different sera from healthy blood donors not reacting with *Toxocara* E/S-antigen in ELISA.

Enzyme-linked immunosorbent assay (ELISA): Two different ELISA systems were used as described by Gottstein et al. (1983) (Zürich) and Speiser (1982) (Basel). The protocols of the test procedures are summarized in Table 1.

Table. 1. Test conditions of ELISA in Basel and Zürich

Steps	Basel	Zürich
Antigen concentration for sensitizing ELISA plates as determined by a previous checkerbord titration	0.3–0.5 μg/ml in coating buffer (0.06 M carbonate buffer solution, pH 9.6)	0.3–0.5 μg/ml in coating buffer (0.06 M carbonate buffer solution, pH 9.6)
Coating and storage of ELISA plates	Coating at 4° C for 24 h and keeping the wet plates at 4° C	Coating at 4° C for 12 h and freezing the plates at −80° C until use
1. incubation	Not done	30′ at 37° C with PBS containing 0.5% bovine serum albumine, 0.05% Tween 20 and 0.02% NaN_3, pH 7.2
2. incubation	Sera diluted 1:160 in PBS (pH 7.2) containing 0.05% Tween 20, 15′ at 37° C	Sera diluted 1:200 in the same buffer solution as above for 2 h at 37° C
3. incubation	Horse-raddish peroxidase labelled anti-human IgG (H+L) produced in goats (Miles No. 61–230), 15′ at 37° C	Anti-human IgG (Fc) produced in rabbits (Dako No. A-089) coupled to alkaline phosphatase (Sigma No. P 6774) according to the method of Engvall and Perlman (1972). Incubation for 2 h at 37° C
4. incubation	Substrate prepared in 0.15 M PBS (pH 5.0) containing 0.1% o-phenylendiamine (Merck No. 8597) and 0.03% hydrogen-peroxide for ±4′ at room temperature	Substrate prepared with nitrophenyl-phosphate (Merck No. 6850) in 0.05 M carbonate buffer solution + 1 mM $MgCl_2$, pH 9.8, for 10′ at 37° C
Stopping of the enzymereaction	50 μl of 8 N sulfuric acid per well was added after the determination of the stopping time according to the expected value of the positive control pool	50 μl of 3 N sodium hydroxide per well
Recording of results	Extinction values at E_{492nm} Extinction values were interpreted according to Speiser (1982)	Reading the optical density at OD_{404nm} The results were recorded as a percentage of the extinction value of a positive reference serum, which was set at 100%
Note	All wells were filled with 200 μl solutions each	Coating was performed with 250 μl solution per well. Subsequent filling of wells with 200 μl solutions each

Table 2. Means and standard deviations of extinction values, obtained with human sera and two *Toxocara canis* E/S-antigen preparations in the BS-ELISA system

Sera	N	*T. canis* E/S-antigen preparation from	
		Basel	Zürich
		Extinction value ($= E_{492nm}$)	
		$\bar{x} \pm s(x)$	$\bar{x} \pm s(x)$
Children without history of toxocariasis (5–15 months old)	20	0.11 ± 0.09	0.11 ± 0.09
Villagers of Jura:			
all	47	0.35 ± 0.45	0.53 ± 0.67
negative[1]	34	0.17 ± 0.10	0.22 ± 0.10
positive[2]	13	0.86 ± 0.62	1.33 ± 0.85
Patients suspected for *Toxocara* infections	25	1.15 ± 0.60	1.41 ± 0.73

[1] $E_{492nm} < 0.50$ with both antigen preparations
[2] $E_{492nm} \geqslant 0.50$ with one or both antigen preparations

Statistical methods: The correlation between extinction values (r) of the two E/S-antigens of ELISA tests were determined according to the estimations of a linear correlation factor and the Spearman rank correlation coefficient (Sachs, 1982; Colquhoun, 1971). The test-reproducibilities were estimated with the calculation of the coefficient of variation (CV):

$$CV\% = \frac{s(x)}{\bar{x}} \cdot 100.$$

Results

1. SDS-PAGE, "Western-blot" technique

SDS-analysis of the two *T. canis* E/S-antigens from Zürich (ZH) and Basel (BS) revealed quantitative and qualitative differences in the correspondingly stained protein patterns. These differences were predominant in the range of 30–90 K daltons (Fig. 1). All bands observed with SDS-PAGE were immunogenic as revealed with the "Western-blot" technique. Some additional bands, which could not be detected by SDS-PAGE, were found by the "Western-blot" method in the BS-antigen. The protein pattern between 94 and 116 K daltons was identic for both antigen preparations from Zürich (3 bands) and Basel (3 bands) as found with SDS-PAGE and the "Western-blot" techniques. No immunoreactive bands were found in control experiments which included a negative human serum pool and concentrated medium (MEM).

2. Distribution of extinction values of various patients' sera

Twenty sera from young children without a history of toxocariasis were tested with the BS-ELISA system using both ZH- and BS-antigens. This investi-

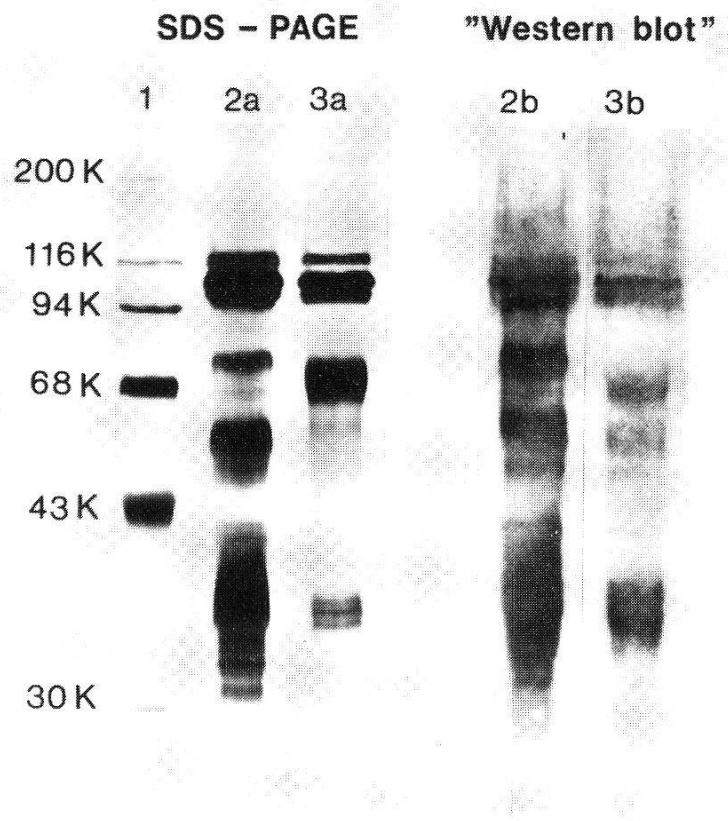

Fig. 1. SDS-polyacrylamide gel electrophoresis and "Western-blotting" of *T. canis* E/S-antigens. Note: The numbers next to the marker bands (1) represent the molecular weights (daltons indicated). a = SDS-PAGE, b = "Western-blot", 2 = antigen preparation from Zürich, 3 = antigen preparation from Basel.

gation served to obtain the range of extinction values with negative sera and to estimate the cut-off between "negative" and "positive" reactions in ELISA. We took $\bar{x} + 4$ S.D. of the extinction values from these children to set the upper limit of negativity at an extinction value of $E_{492nm} = 0.50$ for both antigen preparations (Tab. 2).

In a second experiment, 47 sera from healthy blood donors from a village in a rural area (Southern part of Jura, Switzerland) were tested against the ZH- and BS-antigens in the BS-ELISA system. High extinction values exceeding $E_{492nm} = 0.5$ (see above) were found with both antigen preparations (13 of 47 sera). The accordance of positive reactions with both antigens was 85% (Fig. 2). However, the extinction values with the ZH-antigen were generally higher ($r = 0.959$; Spearmann rank test $2p < 0.01$) than with the BS-antigen (Tab. 2).

With regard to the serodiagnosis of human visceral larva migrans, the scale of extinction values was defined as *negative* from $E_{492nm} = 0.00$–0.49 according to the cut-off defined above (Tab. 2). A *borderline* ranging from $E_{492nm} = 0.50$–0.69 and a *positive* reaction zone exceeding $E_{492nm} = 0.70$ are based on earlier findings (Speiser, 1982).

Under these prerequisitions, 25 lyophilized sera of patients suspected for

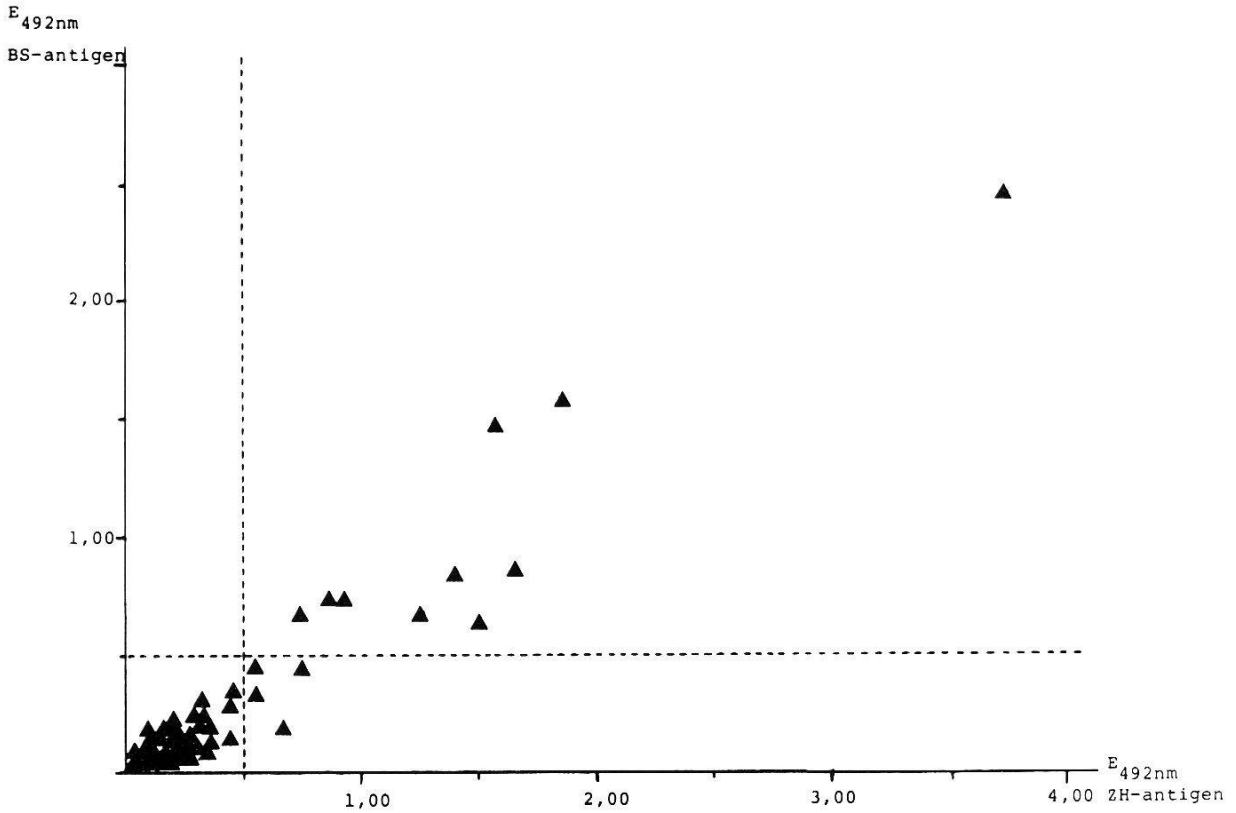

Fig. 2. Distribution of individual extinction values of 47 sera from Swiss villagers (Jura) tested against ZH-antigen and BS-antigen with the Basel-ELISA system. Note: The dotted lines indicate the $\bar{x}+4s(x)$ obtained with sera from 20 children without a history of visceral larva migrans.

toxocariasis with previously positive reactions against the ZH- and BS-antigens in the BS-ELISA system were tested. The extinction values obtained with the two antigens showed a good correlation ($r = 0.737$). This correlation was highly significant as confirmed by the Spearmann rank test ($p < 0.01$). The extinction values were again higher with the ZH-antigen than with the BS-antigen (Tab. 2). Analysing the same sera independently with the ZH- and the BS-ELISA systems and their corresponding antigen preparations, identical serodiagnostic results were obtained in 80% of cases (20 of 25 cases) (Fig. 3). The linear correlation factor ($r = 0.775$) for these two tests was highly significant (Spearmann rank test $p < 0.01$).

3. Specificity

Data collected in 1982 during the routine examination of 3396 human sera with the BS-ELISA were statistically analysed. As indicated in Fig. 4, 21% and 18% of all sera reacting with *T. canis* E/S-antigens ($E_{492} \geqslant 0.50$) were also reactive with crude antigen extracts of *Dipetalonema vitae* and of *Echinococcus granulosus*, respectively (see Speiser, 1980). On the other hand, 13% and 17% of sera from patients with filariasis and echinococcosis, respectively, were reactive with *T. canis* E/S-antigens. This relatively low cross-reactivity differs from the situation among the non-specific *D. viteae* and *E. granulosus* system, where

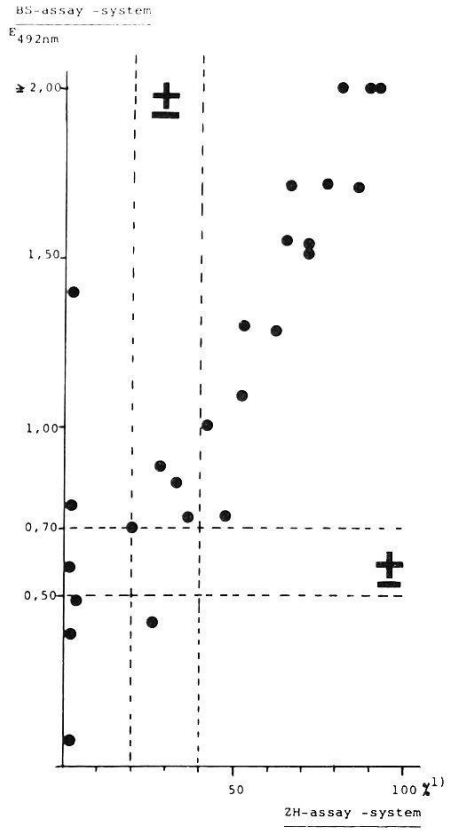

Fig. 3. Comparison of ELISA results with 25 sera from patients with suspected toxocariasis, tested independently with the ELISA system of Basel and Zürich, and their corresponding antigen preparation. Note: The border-line ranges of the two ELISA-systems are indicated by (±). [1]results are expressed in percentages of the optical density (OD_{404nm}) values obtained with a positive reference serum.

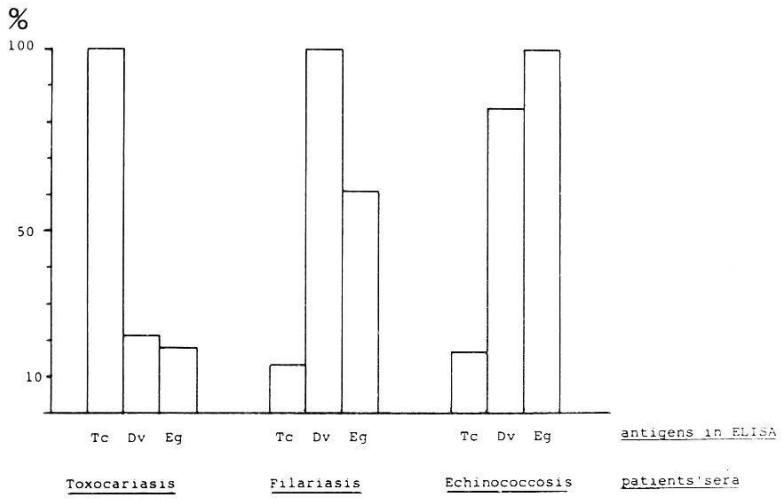

Fig. 4. Analysis of positive reactions from 3394 sera investigated during the diagnostic work of 1982 with *T. canis* E/S-antigen, total worm extract antigen of *D. viteae* and hydatid fluid antigen of *E. granulosus* according to Speiser (1982). Note: All sera reacting with their homologous antigen ($E_{492nm} \geqslant 0.50$) were set as 100% and the corresponding percentages of reactions with heterologous antigens determined.

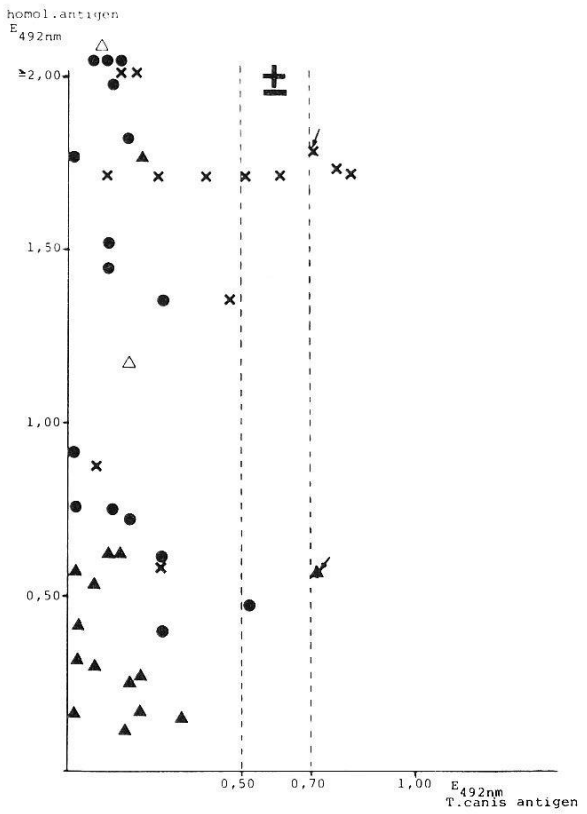

Fig. 5. Individual extinction values of sera from patients with parasitologically proven helminthiasis, tested against their homologous antigens and *T. canis* E/S-antigen with the Basel ELISA-system. Note: sera from patients with schistosomiasis (n = 15) ▲, filariasis (n = 13) ×, echinococcosis (n = 16) ●, fascioliasis (n = 2) △. One serum was from a patient with a *S. mansoni/O. volvulus* double infection (↙).

"second" reactions are encountered much more frequently (60% resp. 83% of the sera, Fig. 4).

Among the 3396 sera tested in 1982, 46 were from patients with parasitologically proven extraintestinal helminthiasis. Individual extinction values of these sera reacting with their homologous antigens and with the *T. canis* E/S-antigens are shown in Fig. 5. Some border-line (3 cases = 6.5%) or weak positive (3 cases = 6.5%)[3] reactions were observed with the *T. canis* antigen. These reactions mainly occurred with sera from patients with filariasis (5 cases) exhibiting high extinction values with homologous antigen. Cross-reactions were not found with *T. canis* E/S-antigen at $E_{492nm} \geqslant 1.00$.

4. Reproducibility

The reproducibility of ELISA with the two antigen preparations from Zürich and Basel were evaluated under routine conditions with the BS-ELISA system. During a period of two months, 500 sera were examined. The analysis of the results revealed the following overall coefficients of variation (CV) (inter- and intra-test variation), expressed in percentages (CV%): 13% with the ZH-

[3] One cross-reacting serum was from a patient with a *S. mansoni/O. volvulus* double infection.

antigen and 15% with the BS-antigen as evaluated with a positive serum control pool. The corresponding mean and standard deviations (n = 70) of E_{492nm} were 1.16 ± 0.15 for the ZH-antigen and 1.07 ± 0.15 for the BS-antigen.

In the BS-ELISA the inter- and intra-test variations were 5.4 CV% and 8 CV%, respectively (n = 40). The corresponding values for the ZH-ELISA (n = 40) were 11 CV% and 12 CV%. The same positive and negative serum pools were used in both laboratories.

Discussion

Second stage larvae of *T. canis* were maintained in a protein free culture medium as described by de Savigny (1975). Results obtained with two culture derived E/S-antigens independently prepared in two laboratories (ZH-antigen, BS-antigen) revealed qualitative and quantitative differences in protein patterns as demonstrated by SDS-PAGE and "Western-blotting". Additionally it was demonstrated that all protein bands resolved by SDS-PAGE were immunogenic (Fig. 1). Ten (BS-antigen) to over 14 (ZH-antigen) different antigenic components were detected. These differences may be due to the following facts: 1. the eggs of *T. canis* were not collected from the same dog. Therefore, intraspecific strain variations of *T. canis* cannot be excluded; 2. since only the BS-antigen was ultra-centrifuged, membrane-bound or aggregated proteins may have been responsible for the additional bands found in the ZH-antigen; 3. quantitative differences of single antigen components as revealed in Fig. 1 may also be responsible for serodiagnostic differences. With regard to a future standardization of *T. canis* E/S-antigen production, the influence of the parameters mentioned above should be clarified.

It remains an open question whether or not all these proteins were adsorbed on the polystyrol surface of ELISA microplates. However, since identical protein concentrations of ZH- and BS-antigen were used to sensitize ELISA microplates it seems likely that the differences found by the SDS-PAGE and "Western-blot" techniques between these two antigen preparations were responsible for the differences found in the serodiagnostic investigations. We compared the two antigens with one ELISA system and found that the ZH-antigen always revealed higher extinction-values with positive sera than the BS-antigen. We obtained a good agreement in serodiagnostic results using the ZH- and BS-antigens, despite of the immunochemical differences.

As no relevant parasitologically proven positive control group was available we determined the diagnostic limits by testing negative sera from children without a history of toxocariasis calculating $\bar{x} + 4s(x)$ for the borderline and $\bar{x} + 6s(x)$ for the positive cut-off and took into account earlier experiences (Speiser, 1982). De Savigny et al. (1979) took 3 times the median "normal" ELISA value as indicative for an earlier acquired or asymptomatical *Toxocara* infection and 8 times the "normal" median value as indicative for an active

toxocariasis. A cut off corresponding to 3 times the "normal" median ELISA value was also used by Matsumura and Endo (1982). Glickman et al. (1978) calculated predictive values by testing 110 sera from presumed toxocariasis patients. A decisive statement concerning the sensitivity cannot be done before a statistically relevant set of sera obtained from patients with toxocariasis becomes available.

All authors working with *T. canis* metabolic antigens and ELISA report a good specificity of the test which agrees with our findings. Using the positive threshold $E_{492nm} = 0.70$ a specificity of 93% was found (Fig. 5). Cross-reactions were observed mainly in sera of patients with filariasis revealing very high titers in their homologous ELISA system. None of the sera with a low or medium titer in its homologous antigen system cross-reacted in the toxocariasis-ELISA. Several authors reported a minor cross-reaction rate. However, in these studies they didn't take into account homologous specific antibody concentrations. In our study, we demonstrated that only sera revealing very high extinction values in their homologous ELISA system cross-reacted in the heterologous toxocariasis system. Furthermore the cross-reactions of these sera with *T. canis* E/S-antigen were always below $E_{492nm} = 1.0$. Therefore we associated extinction values with *T. canis* E/S-antigen higher than $E_{492nm} = 1.0$ with parasitological evidence of toxocariasis.

We assume that antigens of *T. canis* stimulating B-cell-activity may be very species specific. This is supported by the fact that patients reacting with *T. canis* E/S-antigens often do not react with other helminth antigens (Fig. 4), even if these are non-specific, e.g. crude hydatid fluid from *E. granulosus* and total worm extracts of *D. viteae*. The low specificity but high sensitivity of these two antigens have already been described (Bartlett et al. 1975; Speiser, 1980; Weiss et al., 1981). In addition, sera reacting with those non-specific antigens in ELISA generally do not react with metabolic antigens of *T. canis*.

The *T. canis* E/S-antigen appears suitable to be used as a standardized antigen in serology of human toxocariasis. A good reproducibility of ELISA results was demonstrated in this study using two different ELISA systems and antigens prepared independently in two different laboratories. However, variations of serological data observed require further studies in order to obtain precise data about the nature of possible variations of antigen quality. Such studies are in progress and will be published later.

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