

The estimation of different ELISA procedures for serodiagnosis of human trichinellosis

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ABSTRACT. Introduction. The most important confirmative diagnostic test for trichinellosis is the presence of the muscle larvae in a tissue biopsy but this direct method has a low sensitivity of light and moderate infections. The aim of presented study was to compare the usefulness of the results obtained by three ELISA procedures for *Trichinella* spp. diagnosis in human outbreaks. **Materials and methods.** All sera (cases and controls) were tested for anti-*Trichinella* antibodies (immunoglobulin G) using commercially available Novatec KIT and two other ELISA procedures based on excretory-secretory (ES) antigens on *Trichinella spiralis* muscle larvae. The main differences in ELISA procedures were: the protein concentration in antigen, dilution of human serum samples, conjugate and the time of conjugate incubation. Additional differences were noticed in ES antigen preparation procedures as well as in *T. spiralis* isolates used in these procedures. Serum samples were obtained from 22 symptomatical patients from Poznań region (West Poland), geographic area where human outbreak had occurred. Control serum samples were obtained from 20 patients from an open population from a non endemic trichinellosis area. **Results.** The results were analyzed in terms of both: statistical and epidemiological point of view. Linear regression analysis and correlations coefficient *r* between OD values of total 22 patients obtained in three ELISA procedures were positive and high statistically significant. Three ELISA procedures revealed different cut-off values and positivity rates for outbreak. However, the majority of positive samples were found as positive in three procedures, but some of them were positive in two or one procedure only. These individual variability in sera reactivity observed in three ELISA procedures could be very important from epidemiological point of view.

Key words: ELISA procedure, excretory-secretory antigen, human outbreak, *Trichinella spiralis*.

Introduction

Trichinellosis is a worldwide zoonotic parasitic disease caused by the ingestion of raw or undercooked pork or wild game products containing larvae of nematode *Trichinella* [1, 2]. Although veterinarian control measures have been implemented, this disease is still a significant problem in public health in many countries of the world, including Poland [3]. Small outbreaks due to wild boar meat are occasionally reported in hunters and their families in Poland [4, 5]. Trichinellosis can be misdiagnosed with several other diseases because similar clinical symptoms are developed by infected individuals [6, 7]. Human trichinellosis causes very high fever, facial oedema, myositis and eosinophilia. Additionally, it can be a serious disease, particularly in older patients in whom neurological or car-

diovascular complications can lead to death [8, 9].

Up to date, the most important confirmative diagnostic test for trichinellosis is the presence of the encysted muscle larvae in a tissue biopsy [10]. However, this direct method has a low sensitivity of light and moderate infections. Low-grade infections that often cause human trichinellosis can be detected only by more sensitive methods. Of these, ELISA has been demonstrated to be the most sensitive, specific and simple to perform [11-15]. The reliability of serological diagnosis assays highly dependent on the quality and the specificity of the *T. spiralis* antigens used. ELISA procedures had been developed for trichinellosis using excretory-secretory or soluble total extracts of muscle larvae as antigen, mostly because this stage of parasite is easy to obtain [10, 16, 17].

The aim of the present study was to compare the diagnostic efficacy of three different ELISA procedures for human sera from outbreak in area endemic for trichinellosis.

Materials and methods

Serum samples

Serum samples were obtained from 22 symptomatical patients from Poznań region (West Poland), geographic area where human outbreak had occurred. The outbreak consisted of 17 men and 5 women whose age varied from 20 to 75 years. The first clinical symptoms of infection were observed between 10 and 17 day after meet consumption. The patients were hospitalized between 20 and 25 day after infection.

Control serum samples were obtained from 20 patients from an open population from a non endemic trichinellosis area (central part of Poland).

All sera were stored at -20˚C until use.

Parasitological examinations

The presence of *T. spiralis* L1 in home made sausages from wild boars meet was tested using recommended digestive method. The weight of four examined samples ranged from 32 to 54 g.

ELISA procedures

All sera (cases and controls) were tested for anti-*Trichinella* antibodies (immunoglobulin G) using commercially available Novatec KIT and two other ELISA procedures based on excretory secretory (ES) antigens on *Trichinella spiralis* muscle larvae.

Procedure 1: *Trichinella spiralis* IgG-ELISA (Novatec, Immunodiagnostica GMBH, TRIG0480) was used for serological detection of specific antibodies against *Trichinella spiralis* in human serum.

— Dilution of human sera 1/100 in serum *Dilution buffer 2*: 100 µl/well, 1 hour incubation at +37˚C (+/- 1˚C).

— Washing of wells 3 times with *Wash-solution*.

— Addition conjugate: horseradish peroxidase-conjugated *Trichinella spiralis* protein A: 100µl/well, 30 minutes incubation at room temperature.

— Washing of wells 3 times with *Wash-solution*.

— Addition 100 µl/well of 3,3',5,5' tetramethylbenzidine-hydrogen peroxide substrate solution: 15 minutes incubation at room temperature in the dark.

— Addition 100 µl/well of *Stop Solution*.

— Reading of plates, within 30 minutes, for colour density at 450/620 nm on an automated microplates reader (Bio-Tek Elx 800).

Procedure 2 (performed in Physiopathology Laboratory, Witold Stefanski Institute of Parasitology of the Polish Academy of Sciences)

— Coating overnight at +4˚C of the ES Ag: dilution in Coating Buffer (phosphate buffer saline, PBS, pH=7.2) (PBS) at 5 µg/ml: 100 µl per well in 96-wells microtitre plates.

— Washing of wells 3 times with (PBS+0.05% Tween 20).

— Dilution of human sera 1/100 in Diluent Buffer (PBS+0.25% BSA+0.05% Tween 20), 100 µl/well, 90 minutes incubation at +21˚C (+/- 3˚C).

— Washing of wells 3 times with PBS+0.05% Tween 20.

— Addition conjugate Peroxidase *conjugated Rabbit IgG Fraction to human IgG*.100µl/well of 1/40 000 dilution in Diluent Buffer, 60 minutes incubation at +37˚C in the dark.

— Washing of wells 3 times with PBS+0.05% Tween 20.

— Addition 100 µl/well of 3,3',5,5'tetramethylbenzidine-hydrogen peroxide substrate solution, 10 minutes incubation at room temperature +21˚C (+/- 3˚C) in the dark.

— Addition 50 µl/well of *Stop Solution* (40% HF).

— Reading of plates, within 1 hour, for colour density at 630 nm on an automated microplates reader (Bio-tek ELx800).

Procedure 3 (performed in Department of Parasitology, University of Veterinary and Pharmaceutical Sciences, Brno)

— Coating overnight at +4˚C of the ES Ag: dilution in Coating Buffer (phosphate buffer saline,

PBS, pH=7.2 at 2.5 µg/ml: 100 µl per well in 96-wells microtitre plates.

— Washing of wells 4 times with PBS-Tween 20 (PBST+1% BSA).

— Dilution of human sera 1/100 in serum *Dilution buffer 2*:100 µl/well, 60 minutes incubation at 37˚C.

— Washing of wells 3 times with (PBS+0.05% Tween 20).

— Addition conjugate *Peroxidase conjugated Rabbit IgG Fraction to human IgG* 100 µl/well of 1/40000 dilution in, 60 minutes incubation at 37˚C in the dark.

— Washing of wells 4 times with -Tween 20 (PBST + 1% BSA).

— Addition 100 µl/well of 3,3',5,5' tetramethylbenzidine-hydrogen peroxide substrate solution (TMB Complete): 10 minutes incubation at room temperature +21˚C (+/-3˚C) in the dark.

— Addition 100 µl/well of *Stop Solution* (0.5 M H_2SO_4).

— Reading of plates, within 1 hour, for colour density at 450 nm on an automated microplates reader (Bio-Tek Elx 800).

Preparation of excretory-secretory (ES) antigen from L1 muscle larvae

Three ELISA procedures based on ES *T. spiralis* muscle larvae antigen prepared in different laboratories. However the procedure for antigen preparation used in Novatec KIT is unknown.

For procedure 2 ES antigen was prepared in Physiopathology Laboratory, Witold Stefański Institute of Parasitology of the Polish Academy of Sciences. Briefly: after artificial digestion *T. spiralis* muscle larvae (code ISS003) were washed with RPMI-1640. Then larvae were resuspended at 5 000 L1/ml in RPMI-1640 supplemented with 20 mM HEPES, 200 mM L-Glutamine, 100 mM Na-pyruvate and 50 Unites each/ml Penicillin/Streptomycin and incubated in a T-75 culture flask in 5% CO_2 at 37˚C for 18 hr. After that larvae were collected from the medium by settling in 50 ml conical tubes. The medium was filtered through a 0.22 µm filter and the sample was dialysed in PBS pH 7.2 overnight using dialysis membrane with a filter rating of 3 500 kD (Roth, cat. no. E657.1). The protein concentration of the supernatant was determined by Bradford's method [18]. Supernatant was concentrated 100 x by the lyophilization process.

For procedure 3 ES antigen was prepared on Department of Parasitology, University of Veterinary and Pharmaceutical Sciences, Brno. Briefly: the excretory-secretory (ES) antigen from *T. spiralis* (code ISS 1028) was isolated from larvae by *in vitro* cultivation. Muscle larvae recovered from CD1 mice and Wistar rats by artificial digestion were washed seven times in saline. 40 000 larvae were then placed into 250 ml flask with RPMI 1640 medium (with L-glutamine, Sigma Chemical Company, US, No. R-4130) supplemented with 2.5 ml of HEPES, 5 ml of antibiotics (50 IU /ml of penicillin, 50 µg/ml of streptomycin and 100 µg/ml of antimycotics) and incubated for 20 hours at 37˚C in 5% CO_2. The medium was replaced twice (after 6 and 13 hours) for the control of larval viability. After 20 hours, the larvae were removed by centrifugation (5 000 x g for 20 minutes). All medium fractions were pooled and subjected to ultra filtration in an Amicon cell with 10 kDa membrane filter for 1 hour and kept at -20˚C until they were used. As a preliminary step in use of ES antigen in the ELISA, a titration was performed to determine optimal antigen concentration.

***Cut-off* values**

The cut-off of Novatec KIT is the mean absorbance value of the cut-off control determinations. Samples are considered positive if absorbance value is higher than 10% over the cut-off.

The cut-off of ELISA was calculated for the two other procedures separately on the biasis of the average OD plus/minus three standard deviations (SD) [19, 20] of 20 serum samples of patients from an non endemic trichinellosis area (central part of Poland).

Statistical analysis

Statistical analysis was carried out with the MINITAB 8.3 programme and Excel XP. Linear regression and correlation coefficients *r* between ODs revealed statitically significant repeatability of results obtained for serum samples from non infected and infected patients [21]. The significance was established at $P<0.5$.

Results

***Cut-off* values**

The cut-off established in Novatec KIT were 0.4. The cut-off values detected by ELISA using two different procedures on 20 human serum samples from an non endemic trichinellosis area were 0.21 for procedure 2, and 0.16 for procedure 3. Table 1 reflects the OD values for individual patients obtained

in two procedures.

Table 1. Individual OD values for non endemic samples, mean values, SD and cut-off of ELISA performed on procedure 2 and 3

No of patient	Procedure 2 (OD)	Procedure 3 (OD)
1	0.159	0.101
2	0.139	0.15
3	0.135	0.088
4	0.158	0.102
5	0.165	0.095
6	0.184	0.091
7	0.124	0.072
8	0142	0.121
9	0.18	0.098
10	0.123	0.08
11	0.157	0.087
12	0.13	0.091
13	0.164	0.105
14	0.153	0.097
15	0.158	0.097
16	0.164	0.083
17	0.157	0.087
18	0.179	0.129
19	0.15	0.094
20	0.193	0.127
Mean value	0.155	0.099
SD	0.019	0.018
Cut-off	0.223	0.161

Statistically significant repeatability of OD values for negative serum samples was found (P<0.5). The correlation coefficient *r* between procedure 2 and 3 for negative samples was 0. 431 (Fig. 1).

Parasitological examinations

The number of recovered *T. spiralis* larvae ranged from 6.5 to 17.8/g of muscle tissue.

ELISA studies

The mean OD values for outbreak obtained by procedure 1 were 0.981, by procedure 2 was 0.285 and by procedure 3 was 0.267. ODs values of 22 individual sera samples varied from 0.1 to 2.54 in procedure 1, from 0.129 to 0.587 in procedure 2 and from 0.115 to 0.552 in procedure 3 (Fig. 2.1, 2.2, 2.3).

The positivity rate (%) for examined outbreak in procedures 1 and 3 were similar (59.1%) and for procedure 2 was 63.6%. Additionally, individual variability in reactivity of examined sera was observed. Only 9 of samples were positive in these three procedures. Further analysis revealed that in procedure 1, one additional positive sample was positive only in this procedure, one was positive also in procedure 2 and two samples were positive in procedure 3. Two additional samples positive in procedure 2 were also positive in procedure 3 and one was positive only in procedure 2. Results revealed that one of positive samples detected in procedure 3 was positive only in this procedure.

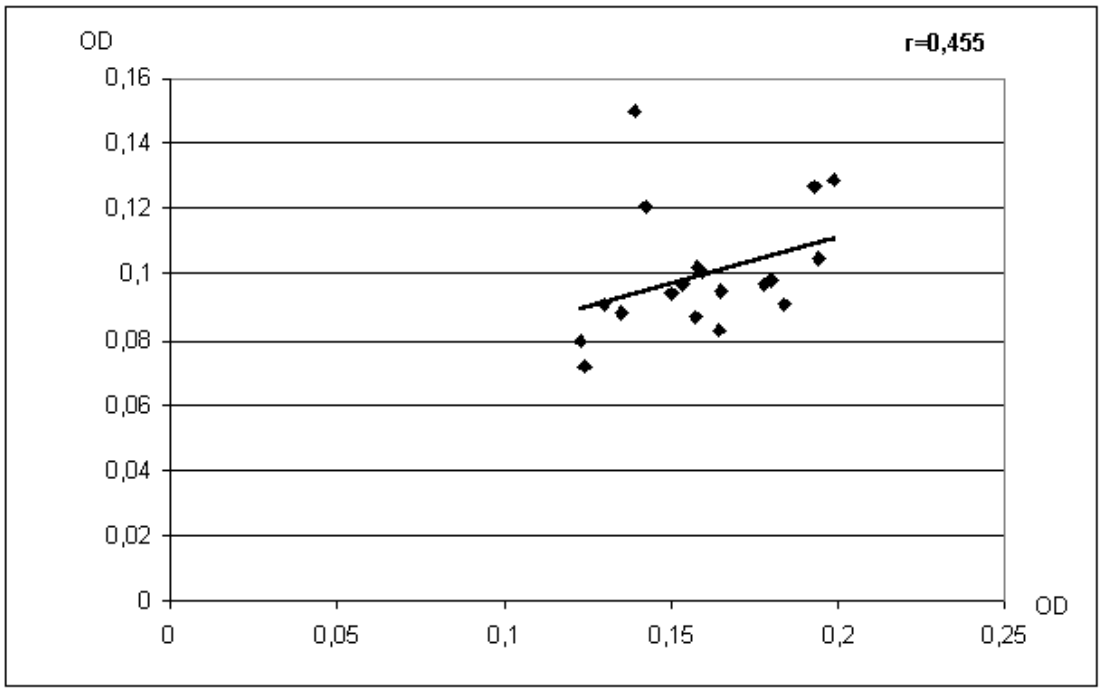

Fig. 1. Linear regression and correlation coeficient for negative samples obtained in procedures 2 and 3

Statistical analysis revealed significant repeatability of OD values for 22 serum samples obtained in three procedures. The correlation coefficient for OD between procedures 1 and 2 was 0.444, between procedures 2 and 3 was 0.817 and between 1 and 3 was 0.568. The linear regression and correlation coefficient *r* for separate procedures are presented on Fig. 3 (A, B, C).

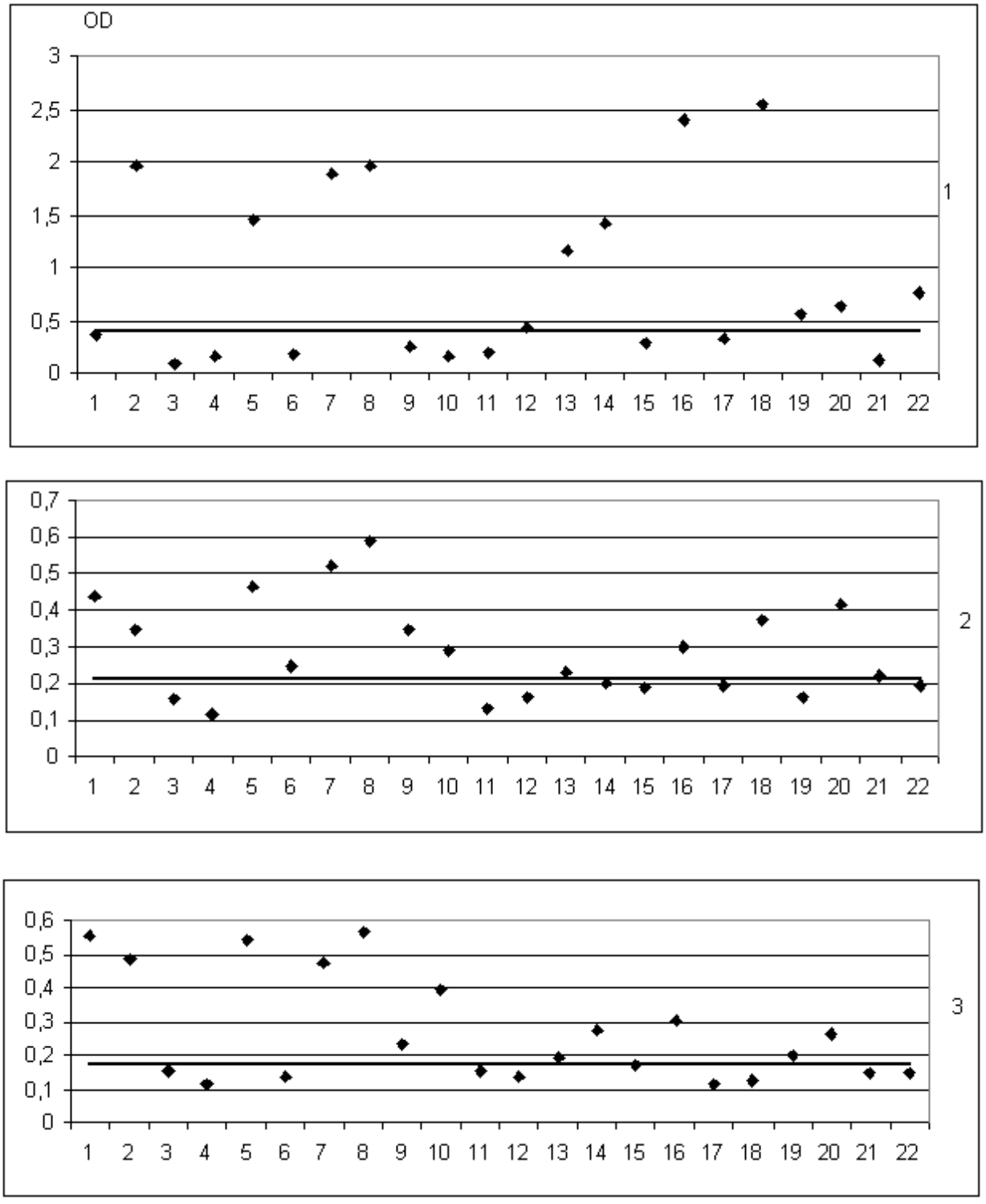

Fig. 2. OD values for samples from outbreak detected in 1, 2 and 3 ELISA procedures,
— *cut-off*; ♦ - OD

Discussion

Since its first application in solid phase assay in the early 1970s, ELISA has become widely used for immunodiagnosis of trichinellosis in both human and swine [11, 19, 22]. In presented studies three ELISA procedures were used to examine IgG antibodies

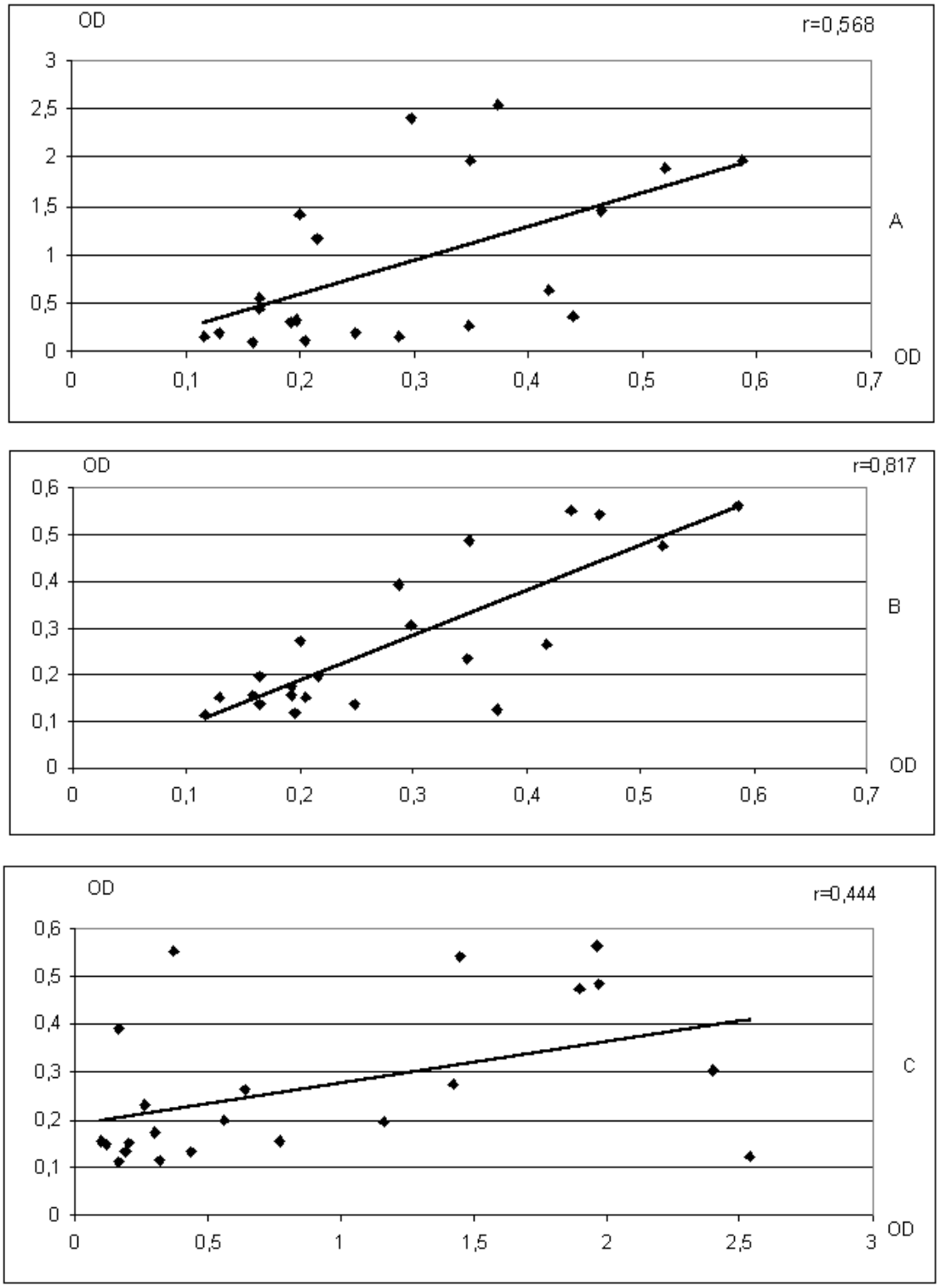

Fig. 3. Linear regression analysis and correlation coeficient for samples from outbreak:
A — procedure 1 and 2; B — procedure 2 and 3; C — procedure 1 and 3

level against *Trichinella spiralis* infection in human outbreak. Basing on the environmental interview and the clinical symptoms, all 22 patients were suspected to be infected with *Trichinella* spp. after wild boars meet consumption. The nematode were isolated from wild boars meet and identified as *Trichinella spiralis* by PCR in Physiopathology Lab. in Witold Stefański Institute of Parasitology of PAS using a multiplex procedure published by authors [23] and confirmed in the Istituto Superiore di Sanita in Rome (Italy), the Reference Centre for *Trichinella* spp.

The main differences in ELISA procedures used in the studies were: *T. spiralis*, excretory-secretory antigen preparation procedure, the protein concentration in ES antigen, the conjugate and the time of incubation with it.

Statistical analysis did not reveal any differences in procedures mentioned above. In our studies all correlations between OD values of 20 control patients from a non endemic trichinellosis area and 22 symptomatical patients obtained in three ELISA procedures were positive and high statistically significant.

Nevertheless the differences mentioned earlier influenced on the quantity of ELISAs results in the epidemiological and/or diagnostics aspects. The highest OD values were observed when the procedure 1 was used. The lower OD values were noticed in procedures 2 and 3, but the similarities in these both procedures reflected the comparable OD values. In all procedures the same human serum dilution was used (1:100), additionally in procedures 1 and 3, plates were incubated with serum samples for 60 min. But in procedures 2 and 3 the same conjugate was used, as well as the same time of incubation. In procedure 1 different conjugate *T. spiralis* protein A and a shorter incubating time were used.

Although the differences in the three ELISA procedures revealed different cut-off values, these did not reflect a slight differences in positivity rates for separate procedures.

While cut-off values for all three procedures were different, the positivity rates for examined outbreak examined by procedure 1 and 3 were comparable (59.1%). The highest positivity rate was found for procedure 2 (63.6%). These parameters strongly reflected some individual variability and reactivity in human samples. The majority of positive samples were found as positive in three procedures. But some of them were positive in two or one procedure only. It is worth to notice that this variability observed for individual human samples could be influenced by *T. spiralis* isolates, protein concentration and the procedures for ES antigens preparation. It is difficult to discuss the influence of these parameters, because the detailed data are available for procedures 2 and 3 only. In these procedures, the 96-wells plates were coated with 100 µl/well of antigen, but the protein concentration was 5 µg/well (procedure 2) and 2.5 µg/well (procedure 3).

In procedure 1 ES antigen was prepared from the isolate coded ISS 003, but in procedure 3, *T. spiralis* isolate coded as ISS 1028 was used. Additional point which differed procedures 2 from procedure 3, was the fact that the medium was replaced twice during *in vitro* cultivation.

In summary, the results obtained for 22 patients suspected to be infected with *T. spiralis* whose sera were examined using three ELISA procedures revealed the highest positivity rates for procedures 2 and lower but similar for procedures 1 and 3. However the individual variability in sera reactivity observed in these procedures could be very important from epidemiological point of view.

It means that variation in a single assay and between-assay must be minimized [24]. To clarify ELISA results in large scale epidemiological studies, the standardization of procedures are needed in all laboratories in both, ES antigen preparation and details of the test protocols.

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