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For in vitro diagnostic use

TK066
ELISA test for the diagnosis of infectious diseases TS (TY.Y 24.4 – 36555928 – 001:2011)

«Vitrotest Anti-Echinococcus»

ELISA test-kit for the detection of antibodies specific to Echinococcus granulosus

1. Intended use

ELISA test-kit «Vitrotest Anti-Echinococcus» is intended for the detection of antibodies IgG and IgA classes to Echinococcus granulosus in human serum or plasma.

The test kit may be used both for the ELISA using automatic pipettes and standard equipment and for setting with the open-system immunoenzymatic automated analyzer.

2. Clinical value

Echinococcosis is a chronic disease of humans and animals caused by parasitizing of helminths Echinococcus larvae. The causative agent of this helminthiasis often is larvae Echinococcus granulosus. Echinococcosis is widespread throughout the world, especially in southern countries, where livestock is spread, mainly sheep farming.

Echinococcus eggs enter the body through dirty hands as a result of contact with dogs (at least - cats). It is also possible to infect when use unwashed vegetables, berries, fruit, water contaminated with eggs of helminths.

In the digestive canal of intermediate host echinococcus egg is released from the shell and the embryo (hexacanth) delves into the mucosa of the small intestine, penetrate through the lymphatic and venous vessels in the portal vein system. As a result hexacanths get to internal organs, where they are, in most cases, retained and developed in echinococcal cysts. Most affected with echinococcosis are liver (in 44-85% of cases), lung (10% of cases), much less kidney, spleen, brain, muscle, etc.

The pathological effect of echinococcosis is caused by sensitization of organism by products of parasite metabolism and mechanical damage to affected organs and tissues. Dimensions of cysts are from 1-5 cm in diameter to large bubbles, which can contain several liters of fluid. Mechanical effects of such cysts lead to dysfunction of the affected organ, its hypertrophy. At the same time, sensitization body by products of parasite metabolism leads to the hypersensitivity of immediate and delayed type.

Echinococcosis often leads to disability, in severe cases to death.

For the diagnosis of echinococcosis are used techniques for imaging cysts: X-ray and ultrasound, computed and magnetic resonance imaging. Needle biopsy of the cyst is considered dangerous because of the possibility of spreading parasites to adjoining tissue.

Given that echinococcosis is accompanied by formation of humoral immune response, serological diagnosis made to the application of invasive procedures gets significant importance. Detection of antibodies specific to echinococcosis antigens in blood is a reliable indicator of parasite infestation. The level of immune response largely depends on the organ localization of the cyst and its morphology. In particular, cysts, localized in the liver, are inducing an immune response more often than cysts in the lungs, brain and spleen; in some carriers of echinococcus cysts specific antibodies are not formed. Low level of antibodies observed at the beginning of the formation of cysts or in the inoperable late stage of the disease.

Today for the detection of antibodies specific to Echinococcus granulosus are using methods of indirect hemagglutination and fluorescence, and enzyme immunoassay. These methods have a sensitivity of 60-90%, so better informativeness is reach using a combination of serological methods.

Quite informative are the serological methods for monitoring the postoperative condition of the patient - a gradual reduction of specific antibodies in 4-6 months after surgical removal of the cyst indicates a favorable outcome of surgery. With recurrent cysts specific antibodies are maintained at a level not high for years.

3. Principle of the test

Principle of the test of «Vitrotest Anti-Echinococcus» kit is based on an indirect enzyme immunoassay technique.

The solid phase is made of strip microplate coated with antigens of Echinococcus granulosus. During incubation of samples in wells of ELISA plate specific to Echinococcus granulosus antibodies are bound to the antigen on the solid phase. After washing out unbound components the mixture of anti-specific conjugates of anti-IgG and anti-IgA monoclonal antibodies with horseradish peroxidase added to the wells, it binds to immune complexes in the solid phase. Unbound components are washed out. The immune complex formed in the wells are visualized by adding chromogen solution of 3,3',5,5'- tetramethylbenzidine (TMB). As a result of the reaction solution in wells where immune complexes were formed would be painted in blue. The reaction is stopped by adding stop reagent, blue colored wells become yellow. The result of the analysis is determined by spectrophotometric reading at 450/620 nm.

4. Materials and equipment

4.1 Contents of the kit

ELISA plate – 12 strips of 8 wells (with the possibility of separation of the wells) with immobilized antigens of Echinococcus granulosus.

Positive control – 1 vial containing 0,3 ml solution of human antibodies specific to Echinococcus granulosus (pink).

Negative control – 1 vial containing 1 ml negative human serum (yellow).

Sample diluent – 1 bottle containing 12 ml buffer with skim milk extract, detergent and preservatives (brown-green).
**Conjugate solution** – 1 bottle containing 12 ml buffer solution of monoclonal antibodies to human IgG and IgA conjugated with horseradish peroxidase, with stabilizers and preservatives (green). Ready to use.

**TMB Solution** – 1 bottle containing 12 ml of TMB solution and hydrogen peroxide, with stabilizers and preservatives (colourless).

**Washing solution Tw20 (20x)** – 1 bottle containing 50 ml 20-fold concentrated phosphate buffer with Tween-20 (colourless).

**Stop-reagent** – 1 vial containing 12 ml of 0,5M sulphuric acid solution (colourless).

**Adhesive film** – 2 sheets of adhesive film for covering the plates during incubation.

**Sera identification plan** – 1 sheet of paper for noting the schemes of samples distribution.

**Instruction for use** – one copy of user manual.

4.2 Additional reagents, materials and equipment

In order to conduct the analysis, the following additional reagents, materials and equipment are required:

- deionized or distilled water;
- filter paper;
- graduated cylinders of 10, 200 and 1000 ml capacity;
- disposable gloves;
- hydrogen peroxide solution 6%;
- disposable glassware for preparing the reagents (bottles and trough);
- timer;
- mono- and multi-channel automatic adjustable pipettes capable of delivering volumes of 20, 200 and 1000 microliters and tips for them;
- thermostat for 37 °C;
- container for solid waste;
- container for liquid waste;
- 1 automatic or semi-automatic washer;
- 2 mono or multi-channel reader for microplates at 450/620 nm.

1,2 Please, consult us about the adaptation of your equipment.

5. Reservations and safety precautions

5.1. Reservations:

- do not use expired reagents;
- do not use in the analysis and do not mix components of different lots and components of test kits with different nosology;
- do not use reagents of other manufacturers in composition with the Vitrotest® sets;

- Note: possible to use washing solution Tw20 (20X), TMB solution and Stop-reagent with other series that are different from those attached to the test kit. These reagents are used in other test systems of Ramintek IPC. Please consult us for details.

- close reagent vials after use only with their appropriate caps;
- strictly follow to the washing procedure requirements described in this instruction;
- control the filling and full aspiration of the solution in the wells;
- use a new distribution tip for each serum and reagent;
- protect kit reagents from straight sun rays;
- TMB solution must be colourless or light blue before its use. If solution is dark blue or yellow, it cannot be used.

Avoid any contact of the TMB solution with metals or metal’s ions. Use glassware thoroughly washed and rinsed with deionized water.

- use only disposable pipette tips for adding TMB-substrate into plate’s wells;
- never use the same glassware for conjugate solution and chromogen.

5.2. Safety precautions:

- all reagents included in the kit are intended for “in vitro” diagnostic use;
- wear disposable gloves when perform analysis;
- do not pipette by mouth;
- the controls of «Vitrotest Anti-Echinococcus» are negative for anti-HCV, anti-HIV1/2, anti-T. pallidum antibodies and HBsAg. Nevertheless, all controls and sera should still be regarded and handled as potentially infectious;
- the liquid waste must be inactivated, for example, with the hydrogen peroxide solution at the final concentration of 6% for 3 hours at room temperature, or with the sodium hypochlorite at the final concentration of 5% for 30 minutes, or with other disinfectant agents;
- the solid waste must be inactivated with autoclaving at 121°C for 1 hour;
- do not autoclave the solutions that contain sodium azide or sodium hypochlorite;
- avoid spilling of TMB-solution and Stop-reagent and any contact of these solutions with the skin or mucosa;
- in case of spilling of solutions, that do not contain acid, e.g. sera, rinse the surface with hydrogen 6% solution, then dry with filter paper.
6. Storage and stability
Reagents of the kit are stable up to the expiry date on the label, when stored at 2-8 ºC.
Transport the test-kit at 2-8 ºC. Disposable transportation at temperature not higher than 20ºC during two days is allowed.

7. Specimen collection
The serum or plasma samples should be stored at 2-8 ºC not more than 3 days after collection. It is possible to store them longer, but frozen (-20 to-70 ºC). Before use frozen samples, wait for 30 minutes for the reagents to stabilize at room temperature. Mix thawed samples well to homogeneity. Avoid repeated freezing/thawing. Samples containing aggregates must be clarified by centrifugation. Do not use samples with contaminated, hyperlipemic and hyperhaemolysed sera.

8. Reagent preparation
It is very important to bring all reagents of the «Vitrotest Anti-Echinococcus» kit to room temperature (18-25ºC) for 30 minutes before use.

8.1. ELISA plate preparation
Before opening the ELISA plate, allow it to stabilize at room temperature for 30 minutes to avoid water condensation inside the wells. Open the vacuum bag and remove necessary amount of wells. Immediately after removal of wells, the remaining strips should be resealed with zip-lock and stored at 2-8 ºC. Microplate in thus packed bag is stable for 3 month.

8.2. Washing solution
The vial contains 50 ml of a concentrated 20X buffer with detergent. Dilute the washing solution 1:20 (1+19) with distilled or deionised water, then mix. For example: for 4 ml of concentrate – 76 ml of distilled water is enough for one strip. Crystals in the solution disappear by warming up to 37ºC for 15-20 min.
The diluted washing solution can be stored at 2-8ºC not more than 7 days.

9. Assay procedure
9.1. Take out from the protective packing the support frame and the necessary number of wells (the number of investigated samples and four wells for controls). Wells with the controls must be included in each analysis.
9.2. Complete the sera identification plan.
9.3. Prepare washing solution (refer to point 8.2).
9.4. Dispense 90 µl of sample diluent in each well.
9.5. Distribute 10 µl of controls and unknown samples into the wells in the following order: A1 – positive control, B1, C1 and D1 – negative control, E1 and rest wells – unknown samples. Gently pipette the mix in wells, avoiding foaming. After addition of serum color of the solution in wells changes from brown-green to blue.
9.6. Cover strips with an adhesive film and incubate for 30 min at 37 ºC.
9.7. After completing the incubation remove the adhesive film carefully and wash the wells five times using the automatic washer or 8-channel pipette in the following order:
- aspirate the content of wells strips into a liquid waste container;
- fill the strip wells with a minimum of 300 microliters of washing solution for each well (respect the soak time of a minimum of 30 seconds);
- aspirate the solution of all wells, the residual volume of solution after aspiration must be lower than 5 microliters;
- repeat the washing step four more times;
- after the last aspiration blot the microplate by turning it upside down on absorbent paper.
9.8. Distribute 100 µl of conjugate solution per well. Cover strips with an adhesive film, incubate for 30 min at 37 ºC.
9.9. After completing the incubation remove the adhesive film carefully and wash the wells five times as described above (refer to point 9.7).
9.10. TMB-solution is ready to use TMB-substrate solution with hydrogen peroxide. TMB-solution should be colorless, protect TMB-substrate solution from straight sun rays. To add TMB-solution only new tips must be used: carefully select a TMB-solution from the vial and without touching the bottom and walls of the hole plate, add 100 µl TMB solution per well.
9.11. Incubate the strips for 30 minutes at room temperature of 18-25ºC in the dark. Do not use adhesive film in this incubation.
9.12. Add 100 µl of stop-reagent in each well. Respect the same distribution sequence than for the TMB-substrate solution.
9.13. Read the optical density of every strip well in dual wavelength reading at 450/620 nm, within the 5 minutes of stopping the reaction. Pay attention to the clearness of the wells bottom outside.
Measurement in the single-wave procedure at 450 nm is possible. Reserve blank well to adjust spectrophotometer in such analysis. Only TMB-substrate and stop-reagent must be added in blank well.
10. Calculation and interpretation of the results

10.1. Test validity conditions:
Calculate the mean optical density (OD) of negative control

\[ OD_{\text{NCmean}} = \frac{OD_{\text{NC1}} + OD_{\text{NC2}} + OD_{\text{NC3}}}{3} \]

In order for an assay to be considered valid, the following criteria must be met:

– OD of positive control is not lower than 0.8 optical unit (OU),
– OD of negative controls should be lower or equal to 0.15 OU,
– OD of every negative control differs no more than twice from the mean value of negative control, that is

\[ OD_{\text{NCmean}} \times 0.5 \leq OD_{\text{NCn}} \leq OD_{\text{NCmean}} \times 2.0 \]

If one of the negative controls does not respect this norm, disregard and recalculate the mean using remaining values.

10.2. Calculation of the results.
Calculate cut-off by adding value 0.30 to the mean NC, that is

\[ \text{Cut off} = OD_{\text{NCmean}} + 0.30 \]

Calculate the index of positivity (IP) for each sample:

\[ IP = \frac{OD_{\text{sample}}}{\text{Cut off}} \]

10.3. Interpretation of the results
The samples with IP above 1.1 are considered positive (IP > 1.1).
The samples with IP below 0.9 are considered negative (IP < 0.9).
The samples with IP within 0.9-1.1 are considered indeterminate (0.9 ≤ IP ≤ 1.1). It is recommended to retest the appropriate samples in duplicate. If the results are again within indeterminate, it is necessary to test sera obtained after 2-4 weeks. If you get undefined results assume that the serum does not contain specific antibodies to Echinococcus granulosus.

11. Limits of the test
A positive result in the test «Vitrotest Anti-Echinococcus» is an evidence of presence in patient of IgG and/or IgA antibodies, specific to Echinococcus granulosus. The presence of antibodies of this class in infants is not evidence of invasion of Echinococcus granulosus.

A negative result in the test «Vitrotest Anti-Echinococcus» indicates the absence of antibodies to Echinococcus granulosus in investigated human serum, or the concentration of specific antibodies is below the sensitivity analysis. That is, a negative result does not rule out the echinococcosis in patient.

The final diagnosis cannot be established only on the basis of serological test. At diagnosis should take into account the complex of laboratory and instrumental studies and clinical manifestations of the disease. It is impossible to completely eliminate cross-reactions with antibodies to antigens of other helminthes. In addition, carefully should interpret the results of studies in patients with cancer and disorders of the immune system.

After successful surgical removal of the cyst, level of specific antibodies begin to decline after 4-6 months.
Reference sources
Legend

Interpretation of notation conventions:

- **IVD**: For in vitro diagnostic use
- **LOT**: Batch code
- **REF**: Catalogue number
- **σ** (Sigma): Production date
- **τ**: Expiry date
- **θ**: Storage temperature limitation
- **Σ**: Meant for <n> tests
- **⊙**: Protect from direct solar radiation
- **⚠**: Attention! Consult instruction for use
- **заметки**: Manufacturer and its address
- **консультация**: Consult instructions for use

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For questions and suggestions regarding the kit, contact the manufacturer:

Ramintek Innovation-Production Company
03039 Ukraine, Kiev, 7 October 40th Anniversary Av., of. 227 (registered address)
07300 Vishgorod, Kiev region, 19 Sholudenko Str. (factual address)
Tel. +380 44 222-76-72
Scheme of the assay «Vitrotest Anti-Echinococcus»

Keep reagents at room temperature (18-25°C) during 30 minutes

Prepare washing solution, dilute 20x concentrate washing solution Tw20 with distilled water 1:20 (1+19). For example, 4 ml of solution + 76 ml of water

Complete the sera identification plan

Dispense 90 µl of sample diluents into the wells

Dispense 10 µl of controls and patient samples into the wells:
A1 – positive control,
B1, C1, D1 – negative control,
E1 and other wells – patient samples
After dispensing of serum the color in well switches from brown-green to blue

Cover wells with adhesive film, incubate for 30 min at 37°C

Wash wells five times

Dispense 100 µl of conjugate solution (green) into the wells

Cover wells with adhesive film, incubate for 30 min at 37°C

Wash wells five times

Dispense 100 µl of TMB substrate solution into the wells

Incubate the plate for 30 min in the dark at room temperature (18-25°C)

Add 100 µl of stopping solution in each well

Read optical density at 450/620 nm

Calculate the cut-off of the assay «Vitrotest Anti-Echinococcus»:
\[ \text{Cut-off} = \text{OD NC mean} + 0.3 \]

Calculate the index of positivity (IP) for patient samples:
\[ IP = \frac{\text{OD of patient sample}}{\text{cut off}} \]

Interpret the results according to the table:

<table>
<thead>
<tr>
<th>IP value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP_{sample} &gt; 1.1</td>
<td>positive</td>
</tr>
<tr>
<td>0.9 ≤ IP_{sample} ≤ 1.1</td>
<td>indeterminate</td>
</tr>
<tr>
<td>IP_{sample} &lt; 0.9</td>
<td>negative</td>
</tr>
</tbody>
</table>