MATERIALS AND METHODS

The present study was carried on 200 cases during the period from April 2015 to February 2016. Stool samples were collected after obtaining written informed consents from all patients.

Stool samples were collected from diarrheic patients attending Abo El Reesh hospital during the period from April 2015 to October 2015. Diarrhea is defined as the passage of three or more loose or liquid stools per day or more frequent passage than normal for the individual (3 times a day) (WHO, 2013).

All cases were subjected to:

1-Full History

- Age
- Sex
- Residence
- Symptoms
- History of previous contact with animals
- History of receiving immunosuppressive drugs as chemotherapy and corticosteroids.

2- Stool Examination

Fresh faecal samples were collected in clean, labelled and wide-mouthed covered containers.

Each collected sample was divided into two parts:

A-First part was freezed at -20°C for immunological and nanotechniques.

B- Second part preserved in 10% formalin for direct examination and staining.
Each samples was subjected to:

* Macroscopic examination for consistency, colour, odour, blood and mucous.

Microscopic examination: direct smear, concentration technique (merthiolate-iodine-formaldehyde concentration technique (M.I.Fc) and staining methods (M.Z.N) for parasitic detection.

**Parasitological Examination:**

The collected samples were examined by:

**A-Direct smear (Michael et al., 2010):**

About 2 mg of stools was taken by a wooden stick and emulsified in normal saline on a clean microscopic slide and cover slip was put then the slide was examined by 10X and 40X magnifications of the ordinary light microscope.

**B- Concentration Method**

Merthiolate-iodine-formaldehyde concentration technique (M.I.Fc) 
(Blagg et al., 1955).

**Reagents:**

MIF solution consists of:

**Stock1**

- Tincture merthiolate 200 ml
- Distalled water 250 ml
- Glycerin 5 ml
- Formaldehyde 36-40% 25 ml
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NB: Tincture merthiolate consist of:

Active ingredients:
Benzalkonium chloride 0.13%

Inactive ingredients:
Acetone, alcohol, DandC red no. 22, purified water

Stock 2
- Iodine crystals 5 gm
- Potassium iodide crystals 10 gm
- Distalled water 100 ml

9.4 ml from stock 1 and 0.6 ml from stock 2 were taken to form 10 ml MIF solution.

Procedure:

One gram of stools was transerred into a test tube containing 10 ml MIF solution.

The suspention was shaked and strained through surgical gause.

The suspention was centrifuged at 3000 g. for 2 minutes.

-If the suspention was not clear it should be get rid of and MIF solution would be added

The suspention was centrifuged again until the supernatant became clear.

The clear supernatant was removed 4 ml of MIF solution and 4 ml of ether was added.

The solution was centrifuged.

All the contents of the tube was removed except the sediment.

A drop of mixed sediment was placed on a slide, covered and examined under microscope.
C- Staining method

Cold Modified Zeihl Nelseen:

The oocyst of *Cryptosporidium* was tested by using modified acid-fast staining method which was sensitive, specific and differential (*Henriksen and Pohlens, 1981*).

Reagents

1-Methanol

2- Carbol fuchsin

- Basic carbol fuchsin powder 5 g
- Absolute ethanol 100 ml
- Phenol crystals 50 g
- Distilled water 900 ml

Preparation

- The basic carbol fuschin was dissolved in absolute alcohol.
- The phenol crystals was dissolved in water.
- The two solutions were mixed.
- The solution was filtered into a brown bottle and labeled.
- The solution was filtered again before use.

3- Malachite green

Reagents

- Malachite green powder 3 g
- Distilled water 100 ml
Preparation

- The malachite green powder was dissolved in water.
- The solution was filtered into a bottle and labeled.
- The solution was filtered again before use.

4- Acid alcohol

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute HCL</td>
<td>3ml</td>
</tr>
<tr>
<td>Absolute methanol</td>
<td>97ml</td>
</tr>
</tbody>
</table>

Procedure:

a) Faecal smear was made directly from the stool sample.
b) Faecal smear was allowed to air dry.
c) Then fixed in methanol for 3 minutes.
d) It was stained with strong carbol fuchsin for 15-20 minutes.
e) It was rinsed thoroughly in tap water.
f) It was decolourised in acid alcohol for 15-20 seconds according to the film thickness.
g) It was rinsed thoroughly in tap water.
h) It was counterstained with 3% malachite green for 30-60 seconds.
i) It was rinsed thoroughly and air dry.
j) It was examined using 40 X and 100 X objectives.

Immunological Examination

Sandwich ELISA and nano-sandwich ELISA

1-Antigenic extract preparation (O’Handley et al., 1999)

Cryptosporidium oocysts were collected from positive infected samples which were mixed together. The sample was examined again by M.Z.N to confirm the infection and examined again by M.I.F technique.
to exclude other infection. The sample was sieved to remove faecal matter. The sample was kept frozen at -20 °C until purification.

2-Antigenic purification

Purification by diethylaminoethyl Sephadex A-50 (DEAE-Sephadex A-50) ion exchange chromatography:

Principle:

DEAE chromatography is an effective method for separating proteins based on their charge. The DEAE group maintains a constant positive charge that is neutralized by counter ions, usually chloride ions. Other anions are capable of competing for the positive DEAE group (Timanova et al., 1999).

Reagent:

1- DEAE Sephadex A-50 (Pharmacia, Yppsalu, Sweden).
2- Tris-HCl (Bio-Rad, USA).
3- NaCl Salt
4- Chromatography column

Procedure:

Gel swelling

About 200 ml of 0.5 M Tris - HCl buffer at pH 8 was placed in a beaker of 250 ml capacity. 0.5 gm Sephadex A-50 powder was added slowly to the buffer with gently stirring using glass rod and left to complete swelling for 1-2 days at 4°C. 0.5 gm Sephadex A-50 powder was swelled to 22 ml beads. The capacity of 1ml swelled beads was calculated as follows:

The available capacity of DEAE-Sephadex A-50 is 5 gm protein/1 mg powder.
2.5 gm protein/0.5 gm powder,
2.5 gm protein/22 ml swelled beads, and
113 mg protein/1 ml swelled beads.

Buffers preparation

1. Binding buffers (20 mMTris-HCl, pH 6, 5.5, 7.7, 5.8 and 8.3).
2. Eluting buffers (20 mM Tris-HCl and 150 mMNaCl).

DEAE column chromatographic method

The swelled beads suspension was poured in 30x2.5 cm column using a glass rod, avoiding air bubbles trapping.

Following the settling of beads in the column, the surface was covered with the binding buffer. The sample was added versus the binding buffer and its protein content was calculated. The outlet tubing was opened till the sample penetration to the beads then closed again for 10 minutes for antigen binding to the beads.

The outlet tubing was then opened and the beads were washed by 5 bed volumes binding buffer. The protein was eluted by 20 mMTris and 150 mMNaCl under gravity by collecting 2 ml fractions. The protein content was estimated by Bio-Rad protein assay (Bradford, 1976).
3-Protein content determination for the antigen

Principle:

Protein content determination was based on Bradford method. The procedure was dependent on the colour change of Coomassie brilliant blue G-250 dye in response to various concentrations of proteins by using protein assay kit (Bradford, 1976).

Reagents:

a) BSA (bovine serum albumin) (Bio Rad, USA)

b) Dye reagent concentrate (Bio Rad, USA) contained phosphoric acid and methanol.

Procedure:

a- Preparation of the standard protein samples.

Serial dilutions from standard protein as BSA were prepared with distilled water as follows:
b- Preparation of unknown protein content sample

Different dilutions of the unknown protein were prepared as follows:-

<table>
<thead>
<tr>
<th>Protein sample</th>
<th>dist. H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 ul</td>
<td>97 ul</td>
</tr>
<tr>
<td>5 ul</td>
<td>95 ul</td>
</tr>
<tr>
<td>15 ul</td>
<td>85 ul</td>
</tr>
<tr>
<td>20 ul</td>
<td>80 ul</td>
</tr>
</tbody>
</table>

The dye (contained phosphoric acid and methanol) was diluted with 4 vol. of dist. H₂O and 5 ml from the diluted dye were added to each dilution from the standard and the unknown protein. Samples were
mixed, the absorbance of the colour was measured at 595 nm for each tube.

**d- Standard curve preparation and protein content calculation**

According to the standard measurements, the standard curve was planed. From the standard curve, the protein content of the unknown samples was calculated.

**4- Immunization of a rabbit**

A new zealand white male rabbit, weighting approximately 1.5 Kg and about 2 months age, purchased from rabbit research unit (RRU), Faculty of Agriculture, Cairo University. They were examined before the start of the experiments and found free from parasitic infection and used in the production of the antibodies (*Abdel-Rahman and Abdel-Mgeed, 2004*). They were housed in the animal house in Theodore Bilharz Research Institute (TBRI), Giza, Egypt. They were kept for 4 weeks (experiment duration) under standard laboratory care at (21°C, 16% moisture, filtered drinking water with added essential minerals as calcium and magnesium 1 mg/5 liter and vitamin 1 mg/10 liter). Diet contain protein, fat and fiber in the form of pellets purchased from RRU.

Blood samples were collected from healthy rabbit ears before injection and examined with ELISA for checking for *Cryptosporidium* antibodies and cross reactivity with other parasites, according to *Gubadia and Fagbemi (1997)*. The rabbit received an intramuscular injection as 1 mg of *Cryptosporidium* antigens mixed with equal volum of complete Freund's adjuvant (CFA) (Pierce, Rockford.IL, USA). Booster doses (0.5 mg *Cryptosporidium* antigens in equal volum of incomplete Freund's adjuvant (IFA) (Sigma, Egypt) were administered at week 2, 3 and 4 after the initial dose according to *Fagbemi et al. (1995)*. Test blood samples
were withdrawn before the injection of each immunization dose to detect the titer of antibodies produced. The animal was sacrificed after 4\textsuperscript{th} week for blood collection, serum preparation and purification.

5-Serum preparation

The blood were collected in centrifugation tubes. The blood tubes were centrifuged at 2000 r.p.m for 10 minutes. The serum was separated in other tubes. The serum tubes were freezeed until purification.

6- Purification of polyclonal Antibody.

The rabbit's sera were obtained and pAb fraction was purified by 50\% ammonium sulphate precipitation method and caprylic acid treatment.
A- Ammonium sulfate precipitation (*Nowotny, 1979*)

**Principle:**

Proteins in solutions form hydrogen bonds with water through their exposed polar and ionic groups. When high concentration of highly charged ions such as ammonium sulfate is added, these groups compete with the proteins for binding to water. This removes the water molecules from the proteins and decreases its solubility resulting in precipitation.

**Reagents:**

1- Ammonium sulfate (Sigma, Egypt)
2- Shaker.
3- Centrifuge.
4- PBS.

**Method:**

i- **Preparation of saturated ammonium sulfate solution (SAS):**

one hundred grms of pure ammonium sulfate salt was dissolved in 100 ml of distilled water. After complete dissolution (2 days), a precipitation method was done.

ii- **Precipitation steps:**

Saturated ammonium sulfate solution was added drop wise to rabbit serum to reach 50% saturation, with continuous stirring and then centrifuged for 20 min. at 3500 g in cooling centrifuge at 4°C. Supernatant was discarded and the ammonium sulfate precipitation steps were repeated several times. The final precipitate was dissolved in a suitable amount of 0.01 M PBS, pH 7.2. The ammonium sulfate was removed by dialysis against 0.01 M PBS, pH 7.2 for 72 h at 4°C.
More purification of pAb was performed by 7% caprilic acid method (McKinney and Parkinson, 1987).

B-Purification by caprylic acid treatment (McKinney and Parkinson, 1987)

Principle:

In mildly-acidic conditions, the addition of short-chain fatty acids such as caprylic acid to the serum will precipitate most serum proteins with the exception of IgG (McKinney and Parkinson, 1987).

Reagents:

1- Caprylic acid (Octanoic acid).
2- Magnetic stirrer.
3- Cooling centrifuge.

Method:

7% caprylic acid was added drop wise with slow magnetic stirring for 30 min. on the srum at 4°C. Albumin and other non-IgG proteins were separated and removed in the precipitate following serum and caprylic
acid mixture centrifugation at 1000 g for 30 minutes. Precipitation was discarded, while the supernatant contained nearly pure IgG. Caprylic acid was removed by dialysis against 0.01 M PBS, pH 7.2 for 72 h at 4°C.

Figure (21): Caprilic acid dialysis

The produced IgG pAb appeared in a very high degree of purity except for few serum protein contaminants. The reactivity of anti-Cryptosporidium IgG pAb against Cryptosporidium antigen was measured by indirect ELISA with some modifications from the original method of Engvall and Perlman (1971). The purity of the produced IgG was assayed (Sheehan and Gerald, 1996).

7-Assessment of reactivity of Cryptosporidium antigen by indirect ELISA:

This method was performed, with some modifications from the original method of Engvall and Perlmann (1971).

Wells of polystyrene microtitre plates (96-flat bottomed wells) were coated with 100 μl/well of Cryptosporidium antigen at a concentration of 5 μg/ml in 0.60 M carbonate buffer, pH 7.4 and incubated overnight at room temperature. The plates were washed 3 times with washing buffer 0.1 M PBS, pH 7.4, then blocked with 100 μl/well of 0.1% BSA in 0.1 M
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PBS, pH 7.4 for 1 hour at 37°C. The plates were washed with washing buffer 5 times. One hundred μl of sera were diluted (1/250) in washing buffer were added to each well then incubated for 1 hr. at 37°C. The plates were washed 3 times with washing buffer. One hundred μl of polyvalent-anti-rabbit peroxidase conjugate (Sigma, Egypt) diluted in washing buffer (1/1000) were dispensed into each well and the plates were incubated for 1 hr. at 37°C. The plates were washed 5 times with washing buffer. One hundred μl of substrate solution of O-phenylene diamine dihydrochloride (OPD) were added to each well and the plates were incubated in the dark at room temperature for 30 min. Fifty μl/well of 8 N H₂SO₄ were added to stop the enzyme substrate solution. The absorbance was measured at 492 nm using ELISA reader.

Reactivity of anti Cryptosporidium polyclonal Ab against different parasitic antigens was done by indirect ELISA to exclude any cross reaction (Giardia lamblia, Entamoeba histolytica, Hymenolepis nana, Entrobius vermicularis, Entamoeba coli).

8-Protein content was measured for the antibody according to Bradford (1976).

Principle:

Protein content determination was based on Bradford dye-binding procedure which was dependent on the colour change of Coomassie brilliant blue G-250 dye in response to various concentrations of proteins by using protein assay kit (Bio-Rad, Richmond, CA, USA) (Bradford, 1976), as mentioned previously.

9-Labeling of IgG pAb with Horseradish Peroxidase (HRP) (Periodate Method) was performed according to Tijssen and Kurstak (1984).
Five mg of horseradish peroxidase (HRP) (Sigma, Egypt) was resuspended in 1.2 ml of dist. H$_2$O. 0.3 ml of freshly prepared sodium periodate was added and incubated at room temperature for 20 min. The HRP solution was dialyzed against 1 mM sodium acetate buffer, pH 4 at 4ºC overnight. Polyclonal solution of 5 mg/ml in 0.02M carbonate buffer, pH 9.6 was prepared. The HRP was removed from dialysis tubing and was added to 0.5 ml of antibody solution. The mixture was left to incubate at room temperature for 2 hr.

One hundred μl of sodium borohydride were added and the solution was incubated at 4°C for 2 hr. The HRP-antibody conjugate was dialyzed against 0.01 M PBS, pH 7.2 (Tijssen and Kurstak, 1984).

10-Preparation of Fecal Samples:

Individual fecal samples were processed by mixing the fecal material in a 1:5 proportion with PBS-formalin 5%. The samples were mixed using a vortex to form a slurry and centrifuged at 3000 r.p.m for 30 min at 25°C. The supernatant was recovered and stored at 4°C until used. On the day of use, the fecal supernatants were mixed well by vortex and recentrifuged at 3000 r.p.m for 15 minutes before used (Mezo et al., 2004).

11-Technique of sandwich ELISA:

After several optimization trials following sandwich ELISA originally described by Espino Finlay, 1994 was performed. Microtitration plates were coated with 10 μg/ml of purified pAb, where M carbonate-bicarbonate buffer dispensed as 100 μl and left overnight at room temperature. Plates were blocked by adding 200μl/well of 3% FCS/PBS/T for 1 hour at 37°C, then, 100μl of fecal supernatant samples was added to each well. Plates were incubated for 1 hour at room temperature. Plates were washed with the washing buffer and 100μl/well
of 1:1000 dilution of peroxidase-conjugated pAb (5 g/ml) were added and incubated for 2 hours at room temperature, then the plates were washed as before.

The reaction was visualized by the addition of 100 μl/well of O-phenylenediamine (OPD) substrate solution for 30 minutes in the dark at room temperature. The reaction was stopped by adding 50 μl/well of 8 N H₂SO₄ and plates were read at 492 nm using ELISA microplate reader (Bio Rad, USA).

**Figure (22):** ELISA microplate reader

### 12- Gold nanoparticles:

*Characterization and functionalization*

AuNPs were purchased from Sigma Company for Photo-electronics with average diameter of 20 nm checked with a transmission electron microscope. AuNPs were functionalized according to *Omidfar et al., (2010)*. Briefly, 100 ml tetrachloroauric acids (0.01% w/v HAuCl₄) were brought to boiling, and then a solution of 1% trisodium citrate was added with constant stirring. When the solution’s color changed from light yellow to wine red, after about 8 min, the solution was cooled down. The pH was adjusted to 8.5 using 0.01 M potassium carbonate, and 0.01% (w/v) sodium azide was added. The obtained solution was stored at 4 °C in a dark-colored glass bottle for several months.
**Materials And Methods**

*Loading of AuNPs to pAb (Kumar et al., 2008)*

To prepare either AuNPs-pAb or AuNPs-HRP-pAb briefly, 600 ug of pAb or HRP-pAb (30ug/ml, in phosphate-buffer (pH 7.5) was added to 20ml pH-adjusted AuNPs solution. The mixture was gently mixed for 3 h, and subsequently 4ml of 10% BSA solution was added to block the residual surface of the AuNPs. The mixture was then incubated for 20 min at room temperature before being centrifuged at 3000 r.p.m for 45 min at 4°C for three times. After the last centrifugation, the pellets were re-suspended in 2ml phosphate buffer (pH 7.2, 0.01 M) containing 1% BSA and 0.05% sodium azide). AuNPs-MAb and AuNPs-HRP-MAb were stored at 4°C before being used.

*Optimization of working dilutions of coating AuNPs-pAb and AuNPs-HRP-pAb (Deelder et al., 1989; Demerdash et al., 1995) and determination of the lower detection limit by indirect ELISA*

The prepared AuNPs-pAb and AuNPs-HRP-pAb were tested in different concentrations to obtain optimum dilutions by chequer-board titration against known positive and negative samples. Optical density (OD) readings at 492nm were plotted against the concentrations of the antigen preparations to determine the lower detection limit.

*Technique of nano-sandwich ELISA:*

A novel sandwich ELISA using conjugated pAb with nano-gold beads nanoparticles as the capture antibodies and pAb conjugated with HRP as the conjugated antibodies. Microtiter plates were coated with 1μg/ml of capture antibody, 100μl/well, which was diluted in carbonate-bicarbonate coating buffer, pH 9.6 and the plates were incubated at 4°C overnight. In the next morning, the plates were washed three times with 200 μL of PBS (pH 7.3), then 200 μL of blocking buffer (PBS/0.3%
Tween-20) was added to each well, including blanks. Plates were re-sealed, and left 1 hour at room temperature on an orbital shaker. After that, 100 μl of fecal supernatant samples were added to wells in a duplicate, followed by resealing the plates and incubating them for 1 hour at room temperature on the shaker. Plates were washed as mentioned above. Peroxidase-conjugated antibody was added at 5 μg/mL in blocking buffer (100 μl/well) to all wells, but not the blanks. Plates were re-sealed, incubated for 1 hour at room temperature on an orbital shaker and were washed. The reaction was visualized by the addition of 100 μl/well of O-phenylenediamine (OPD) substrate solution for 30 minutes in the dark at room temperature. The reaction was stopped by adding 50 μl/well of 8 N H₂SO₄ and the plates were read at 492 nm using ELISA microplate reader.

**Figure (23):** Conjugated pAb with nano-gold beads nanoparticles
STATISTICAL ANALYSIS

Key Features in Reliability of Test Results

Assay or test specificity and sensitivity can be selected and adjusted to meet the needs of a clinician for the diagnosis and monitoring of a disease. This may be accomplished by changing the selection of the reference value (i.e., cut-off or upper limit of normal) for the particular test (Zane, 2001).

1- Diagnostic sensitivity

Diagnostic sensitivity of a method refers to the frequency (calculated) of a positive test results detected by a particular method in individuals with a particular disease.

\[
\text{Sensitivity} \%(\%) = \frac{\text{No. of true positive results}}{\text{No. of +ve results} + \text{No. of false -ve results}} \times 100
\]

Therefore, the higher the test sensitivity (\%), the higher is the number of positive results in diseased individuals.

2-Diagnostic specificity

Diagnostic specificity of a method refers to the frequency (calculated) of negative test results detected by a particular method in individuals without the particular disease.

\[
\text{Specificity} \%(\%) = \frac{\text{No. of true negative results}}{\text{No. of -ve results} + \text{No. of false +ve results}} \times 100
\]

Therefore the higher the test specificity (\%), the higher is the number of negative results in healthy individuals.
3- Positive and negative predictive values

Percentage of positive results that are true positive or percentage of negative results that are true negative.

$$PPV (\%) = \frac{\text{No. of true positive results}}{\text{No. of true +ve results} + \text{No. of false +ve results}} \times 100$$

$$NPV (\%) = \frac{\text{No. of true negative results}}{\text{No. of true -ve results} + \text{No. of false -ve results}} \times 100$$

The data are presented as mean ± standard deviation of mean (X ± SD). The mean values of each group were calculated from the mean values of individual patients. The mean groups were compared by analysis of variance (Snedecor and Cochran, 1981). The comparison between various groups was done using either Student's T test or ANOVA. Correlation between the optical density of ELISA technique and human groups was performed by application of correlation coefficient according to Snedecor and Cochran (1981).

The data were considered significant if p values were equal to or less than 0.05. Statistical analysis was performed with the aid of the SPSS computer program (version 6.0 windows) (Lee and Brooke, 1997).