Cryptosporidium species in ducks: parasitological, serological and molecular studies in Egypt.

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Abstract

The present work was initiated to study the incidence, morphology and molecular identification of Cryptosporidium spp. infecting ducks. Incidence of Cryptosporidium spp. in ducks was 39.9 % (365 out of 915 ducks). Seasonal incidence showed that the highest rate was in winter (74.6%) while the lowest one was in autumn (7.1%). Cryptosporidium spp. oocysts were spherical to ovoid in shape with smooth wall. It contains 4 sporozoites and a residuum. Comparative study between M.Z.N. and ELISA showed that examination of the bursal and intestinal contents of each of 100 Muscovy ducks by M.Z.N. stain revealed that 33% and 28% were Cryptosporidium spp. positive respectively. While ELISA showed that 55% of these ducks had antibodies against Cryptosporidium species. Immunoblotting of Cryptosporidium spp. antigens of ducks revealed that the bands at the MW 68, 38-42, 20 and that lower than 15 KDa considered to be specific as they react versus Cryptosporidium positive duck serum only. Analysis of the PCR products of Cryptosporidium spp. from ducks revealed that the positive amplification of the target fragment was 435bp for ducks. Sequencing of the PCR products revealed that the investigated species was C. meleagridis in ducks (the accession number in gene bank was KM581271).

Keywords: Cryptosporidium spp., ducks, incidence, ELISA, immunoblotting, PCR.

Introduction

Ducks are hardy animals and good scavengers. About 700 million ducks are kept around the world. More than 500 million are in Asia while the reminder numbers of ducks are in other parts of the world, including Africa and Latin America (Van der Meulen and Den Dikken, 2004). Ducks can be reared for eggs and meat for own use or for sale. Other products from ducks, which can be sold, include down, feathers, and fattened livers. The ducks of the world are of two types, the Muscovy ducks (Cairina moschata) and the mallard ducks (Anas platyrhynchos) (Harrison and Greensmith, 1993). There are several breeds of ducks in Egypt, Native, Sudanese, White Peckin, Mallard Muscovy and Campbell (AbouLaila et al., 2011).

Cryptosporidium species are one of the most prevalent protozoan pathogen develop in the bursa of fabricius, cloaca, intestine, trachea and kidney of birds (Dubey et al., 1990). It causes diarrhea and unthriftiness, in addition to pathological changes in infected ducklings as epithelial hyperplasia, loss of cilia and dilated mucous glands in trachea. In bursa of fabricius, it causes hyperplasic hypertrophied epithelium, thickened stroma and infiltration of lamina propria with mononuclear cells and heterophils (Mousa, 2000). In birds, three main Cryptosporidium spp. have been described namely C. baileyi, C. galli and C. meleagridis (Slavin, 1955, Current et al., 1986, Ryan et al., 2003). Several researches referred to
infection with another species of Cryptosporidium in birds like C. parvum which was recorded as natural infection in different wild and pet birds (Ryan, 2010 and Graczyk et al., 1996).

C. meleagridis is the third most common Cryptosporidium parasite in human (Xiao, 2010) and has the ability to transfer from infected birds to human (Chappell et al., 2011).

Cryptosporidium spp. among ducks are well documented all over the world (Richter et al., 1994, Adejimi and Oke, 2011, Jasim and Marhoon, 2015). In Egypt there were information only on the incidence and description of Cryptosporidium spp. by light microscope (Abu-Siry, 1993, Abdel-Wanis et al., 1996, Mousa, 2000, El-Madawy, 2001, AbouLaila et al., 2011 and Nagwa et al., 2013) but there is no previous work on the serological and molecular diagnosis. So, the present work was initiated to study the incidence of Cryptosporidium spp. infection in ducks using parasitological [modified Ziehl-Neelsen stain (M.Z.N.)] and serological [Enzyme Linked Immuno-Sorbent Assay (ELISA)] methods, characterization of Cryptosporidium spp. antigens by using Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis (SDS-PAGE) and immunoblotting, molecular identification of Cryptosporidium-positive samples by polymerase chain reaction (PCR) and sequencing of the PCR products.

Materials and Methods

1. Collection of samples:

1. a. Intestinal contents and bursal samples:

Intestinal contents and bursal samples were collected from each of 915 ducks (820 Muscovy, 70 Mallard and 25 Native) during the period from January to December 2014 at age varied from one week to 6 months.

1. b. Blood samples:

One hundred random serum samples were collected from the previously examined 820 Muscovy ducks. The intestinal, bursal and serum samples of these 100 ducks were used for comparative study between M.Z.N. stain technique and ELISA for diagnosis of Cryptosporidium spp. infection.

2. Diagnosis of Cryptosporidium spp.:

2.1. Parasitological examination:

The intestinal contents and bursal samples were stained by M.Z.N. stain (Henriksen and Pohlenz, 1981). The morphological parameters for 50 Cryptosporidium oocysts were measured under light microscope.

2.2. Serodiagnosis of Cryptosporidium spp. in the 100 duck sera by ELISA:

2.2.1. Preparation of antigen:

The Cryptosporidium spp. oocysts used for preparation of antigen were collected from naturally infected ducks. The oocysts were purified according to Khalil, 1993. The protein content of the prepared antigen was measured according to Lowry et al., 1951.

2.2.2. ELISA procedure (Catty and Raykundalia, 1989):

The antigen concentration after checkerboard titration was 3µg protein / well, serum dilution was 1:100 and anti-chicken IgG horse reddish peroxidase (1:1000 dilutions). The optical density was read at 450 nm with a micro-ELISA reader system.

3. Characterization of Cryptosporidium spp. antigens by using Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis (SDS-PAGE) and immunoblotting:

3.1. SDS-PAGE:

Three different types of antigens were prepared from purified Cryptosporidium spp. oocysts of naturally infected ducks, Cryptosporidium baileyi of naturally infected chickens and Cryptosporidium parvum of naturally infected calves. These antigens were electrophoresed by SDS-PAGE according to Laemmli, 1970.

3.2. Immunoblotting:

The unstained protein bands of the previously prepared antigens (ducks, chickens and calves) were electrophoretically transferred from SDS-PAGE to a nitrocellulose sheet using modified technique according to Towbin et al., 1979.
4. Molecular identification of *Cryptosporidium* spp.:

4.1. DNA Extraction:

*Cryptosporidium* spp. oocysts were isolated from the bursa of naturally infected ducks and chickens. The oocysts were purified and stored at -20°C until used for DNA extraction. DNA was extracted by DNA extraction Kits (Qiagen).

4.2. PCR amplification:

PCR amplification for *Cryptosporidium* spp. was processed using 18s rRNA gene and primers (forward and reverse) as shown in Table 1. Cycling protocol used in PCR for identification of *Cryptosporidium* spp. was done according to Johnson et al., 1995. PCR products were placed on 1% agarose gel for electrophoresis and stained with ethidium bromide using 100 bp DNA ladder as size marker (fermentas, Germany): they visualized under UV trans-illuminator and photographed using gel documentation system (Sambrook et al., 1989).

4.3. Sequencing of the PCR products and submission to Genbank:

Purified PCR products were sequenced in both orientations by the dideoxy chain-termination method using the amplification primers which described above. Gene sequencing was carried out using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) at Bethesda, Maryland USA using 3730XL DNA sequencer. The whole experimental process was monitored through LIMS (Laboratory Information Management System). The results of sequencing were analyzed by BLAST web tool of the Genbank (NCBI). http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome. Analysis runs on all releases of Microsoft Windows operating system (BIOEDIT version 4.0.2 software) (Tamura et al., 2007). The results of sequencing were analyzed by BLAST web tool of the GenBank (NCBI). Sequence submission to the GenBank was conducted following the instructions offered by the web tool BankIt of GenBank http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank.

5. Statistical Analysis:

Data on morphological parameters were analysed on the computer statistical package SSPS using Chi-square test. Differences expressed as significant at *P* < 0.05 (Verzani, 2004.).

Results

1. Diagnosis of *Cryptosporidium* spp.:

1.1. Incidence of *Cryptosporidium* spp. infection in the examined 915 ducks by M.Z.N. stain:

The incidence of *Cryptosporidium* spp. in 915 examined ducks was 39.9% and 36.4% in bursal and intestinal samples respectively (Table 2). It is worth to mention that the positive intestinal samples were included in the positive bursal samples. Seasonal incidence showed that the highest rate was in winter (74.6%) while the lowest one was in autumn (7.1%) as shown in table 3. Concerning the age, it was recorded that 66.5% of the examined young age (1W-2M) was infected with *Cryptosporidium* spp. while the older one (> 2-6 M) showed negative results (Table 4).

<table>
<thead>
<tr>
<th>Duck breed</th>
<th>No. of examined</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bursal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ve</td>
</tr>
<tr>
<td>Muscovy</td>
<td>820</td>
<td>340</td>
</tr>
<tr>
<td>Mallard</td>
<td>70</td>
<td>25</td>
</tr>
<tr>
<td>Native</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>915</td>
<td>365</td>
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</table>
1.2. Morphology of the detected Cryptosporidium spp.:  
Light microscopical examination of M.Z.N. stained smears from the bursal and intestinal contents of ducks showed that Cryptosporidium spp. oocysts appeared as acid fast (red-pink) on a green background. The sporulated oocysts were spherical to ovoid in shape with smooth wall. It contains 4 sporozoites and a residium. The measurement of 50 oocysts from M.Z.N. stained smears were 4.5-6.0 µm in length (mean 5.55 µm, S.D. 0.56) and 4.5-5.0 µm in width (mean 4.8 µm S.D. 0.245).

1.3. Results of examination of 100 Muscovy duck samples by M.Z.N. stain and ELISA:  
Examination of the bursal and intestinal contents of each of 100 Muscovy ducks (from 820 Muscovy ducks) by M.Z.N. stain showed that 33% and 28% were infected with Cryptosporidium spp. oocysts respectively. While examination of the sera of these 100 Muscovy duck by ELISA revealed that 55% had antibodies against Cryptosporidium species. All M.Z.N. stain positive samples were out of the 55% ELISA positive samples (Table 5).

2. Characterization of Cryptosporidium spp. antigens by SDS-PAGE and immunoblotting:  
2.1. SDS-PAGE:  
Characterization of the three purified Cryptosporidium spp. antigens by SDS-PAGE revealed that Cryptosporidium spp. of ducks were fractionated into 6 bands at molecular weight (MW) 75, 69, 57-64, 48-53, 22-25 and 18-20 KDa. While C. baileyi of chickens were fractionated into 6 bands at MW 75, 69, 57-64, 55-57, 22-25 and 18-20 KDa. In addition to C. parvum of calves were fractionated into 5 bands at level of 75, 69, 51-53, 22-25 and 18-20 KDa (Fig.1).
2.2. Immunoblotting:

Nitrocellulose (NC) strips containing fractionated Cryptosporidium spp. of duck antigen were treated versus different sera. The data in (Fig. 2) revealed that 12 Protein fraction bands (PFB) corresponding to Molecular weight (MW) 135, 100, 68, 58-62, 38-42, 30-35, 25, 18-20, 17, 15, 12 and 11 KDa (Lane 1) were demonstrated after treatment of NC strip versus infected duck serum. In the same time treatment of similar strip by serum of C. baileyi of infected chickens revealed 7 reacted bands corresponding to MWS >135, 100, 75, 48-62, 35, 30 and 25 KDa (Lane 2) while treatment versus calf infected serum revealed 5 band at MWS 100-135, 75, 48-62, 35 and 32 KDa (Lane 3). In the same time, only 3 bands (MW 32-35, 18, 17 KDa) were recorded in fractionated duck Cryptosporidium spp. versus negative duck serum (Lane 4). From the previous reaction, the bands at MW 68, 38-42, 20 and that lower than 15 KDa were considered to be specific for duck strain as they react versus serum of infected duck only. In the same time some PFB at MW 100, 62, 35and 25 KDa were considered to be common between antibodies in the tested sera. In the same time the fractions at MW 20-38 KDa were considered to be specific for chickens as it react versus chicken C. baileyi infected serum only (Lane 5 & 6). Bands at MW 34, 25 and 16 KDa were considered to be specific versus infected calf serum after treatment of fractionated calf C. parvum (Lane 7 &8).

3. Molecular characterization and sequencing of Cryptosporidium spp. isolated from ducks and chickens:

Analysis of the PCR products on agarose gel electrophoresis revealed the positive amplification of the target fragment of Cryptosporidium spp. with correct size 435bp for ducks and chickens (Fig. 3). Duck and chicken specimens revealed similarity nearly 99% to C. meleagridis and C. baileyi according to accession number in gene bank (KM581271 and KM581270) respectively (Table 6).

Table 6: Genotype of Cryptosporidium by using basic local alignment:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Accession No.</th>
<th>Isolation source</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KM581270</td>
<td>Bursal content</td>
<td>Chicken</td>
</tr>
<tr>
<td>2</td>
<td>KM581271</td>
<td>Bursal content</td>
<td>Duck</td>
</tr>
</tbody>
</table>

Fig.1: SDS-PAGE demonstrated different protein fractions in Cryptosporidium spp. purified oocyst:

Fig. 2: *Cryptosporidium* spp. fractionated antigens reacted versus positive and negative sera using immunoblotting.

**Duck Ag**
- Lane 1: duck Ag versus + Ve duck serum
- Lane 2: duck Ag versus + Ve Chicken serum
- Lane 3: duck Ag versus + Ve Calf serum
- Lane 4: duck Ag versus – Ve duck serum

**Chicken Ag**
- Lane 5: chicken Ag versus + Ve Chicken serum.
- Lane 6: Chicken Ag versus – Ve Chicken serum.

**Calf Ag**
- Lane 7: Calf Ag versus + Ve Calf serum.
- Lane 8: Calf Ag versus – Ve Calf serum.

Fig. 3: Agarose gel stained with Ethidium bromide showing the PCR amplification products of *Cryptosporidium* spp.

Lane M: 100bp DNA size marker; Lane 2: Positive PCR signal for *Cryptosporidium* spp. from chickens (435bp).
Lane 4: Positive PCR signal for *Cryptosporidium* spp. from ducks (435bp).

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Discussion

Cryptosporidiosis was reported in a variety of mammalian species including human as well as avian species as turkeys, chickens, ducks, geese and quails (Fayer and Ungar, 1986). The present study showed that examination of each of 915 duck samples by using M.Z.N. stain technique revealed that 39.9% was Cryptosporidium spp. positive. This result was similar to that reported by Mousa, 2000 (40%) in Egypt. On the other hand, number of authors showed dissimilar results where 26.8%, 24.2%, 16%, 0% and 13.8% was recorded by Abu-siry (1993), El- Madawy (2001), Abdel-Wanis et al. (1996), Abo-Laila et al. (2011) and Nagwa et al. (2013) at different localities in Egypt respectively. Also, our results disagreed with that reported on other countries by Wang and Leiw, 1990 (66%) in Taiwan, Richter et al., 1994 (57%) in Frankfurter, Kuhn et al. (2002) in Southern New Mexico (49%), Majewska et al., 2009 in Malaysia (33.3%) and Wang et al., 2010 (16.3%) in China. The difference between our results and those authors might be attributed to the variation of the number and breeds of examined ducks. Also, might be due to difference in the investigated localities, type of samples, methods of diagnosis, hygienic measures, bird management and environmental conditions. Seasonal incidence showed the highest incidence was in winter (74.6%) while the lowest one was in autumn (7.1%). Our results agreed with Nagwa et al. (2013) in Egypt where the incidence was 44.2% and 2.4% in winter and autumn respectively. This result disagreed with Mousa, 2000 in Egypt and Mohammed (2009) in Iraq whom reported high incidence in summer and spring (15.3% and 84% respectively). While the lowest one was 6.1% and 68% in winter and summer respectively. In relation to age, the rate of Cryptosporidium infection in the present work was 66.5% in young ages (1W-2M). Other authors as Mousa (2000) in Egypt (1-6 weeks), Richter et al. (1994) in Germany (1-5 weeks) and Wang et al. (2010) in China (11-30 day) recorded 30.7%, 57.03% and 40.3% respectively.

The morphological parameters of 50 Cryptosporidium spp. oocysts by M.Z.N. stain were spherical to ovoid in shape with smooth wall. It contains 4 sporozoites and a residium. Its measures varied from 4.5-6.0 x 4.5-5.0 µm. Previous authors not give definite identification morphometrically to the species infecting ducks Mousa (2000), El- Madawy (2001) and Mohammed (2009) where they mentioned that ducks were infected by Cryptosporidium species. The measurements by light microscope not give accurate identification for Cryptosporidium spp. infecting ducks but the identification of the detected species will be confirmed by molecular identification.

Examination of the bursal and intestinal contents of each of 100 Muscovy ducks (from 820 Muscovy ducks) by M.Z.N. stain showed that 33% and 28% were infected with Cryptosporidium spp. oocysts respectively. While examination of the sera of these 100 Muscovy ducks by ELISA revealed that 55% had antibodies against Cryptosporidium species. All M.Z.N. stain positive samples were out of the 55% ELISA positive samples. The incidence of Cryptosporidium by ELISA was higher than that recorded by M.Z.N. stain. This might be due to the ducks were chronically infected so the oocysts not detected due to their low numbers in the bursal and intestinal contents but the immune system was stimulated to release Cryptosporidium antibodies.

Identification of the specific protein fractions using SDS and immunoblotting revealed that the bands at the MW 68, 38-42, 20 and that lower than 15 KDa considered to be specific for duck strain as they react versus serum of infected duck only. In the same time, the fraction at MW between 20-38 KDa in the fractionated chicken antigen considered specific versus C. baileyi infected chicken serum only. While that at MW 34, 25 and 16 KDa in the fractionated calf antigen considered specific versus C. parvum infected calf serum. In the same time protein fractions corresponding to MW 100, 62, 35and 25 KDa appear as common bands of antibodies in the different tested sera. The specific fractions of C. baileyi of chickens recorded in the present study (20-38 KDa) were occurred in the same range previously recorded by Ditrich et al., 1993 (15.5 to 33 KDa). Immunoblotting of C. parvum in the present study showed fractions at MW 34, 25 and 16 KDa. On the other hand, fractions at MW 17-69 KDa and 105-300 KDa were detected by Lorenzo et al. (1995) and Kramer et al. (2007) respectively. The difference in the number of bands with this author may be related to type of used sera. The common bands 100, 62, 35and 25 KDa were considered as cross-reacted band between duck, chicken and calf sera. The difference in bands might be due to difference in genotypes, and level of antibodies in the tested sera.

In the present work analysis of the PCR products on agarose gel electrophoresis revealed the positive amplification of the target fragment of Cryptosporidium with correct size 435bp for ducks and chickens. Also, Duck and chicken specimens resulted nearly 99% homologus to C. meleagridis and
C. baileyi according to accession number in gene bank (KM581271 and KM581270) respectively. Champliaud et al. (1998) identified the same base pair (435) of Cryptosporidium spp, in quail and chickens. While Wang et al. (2010) identified C. baileyi in chicken and ducks by PCR and Bomfim et al. (2013) reported that the Cryptosporidium sequence from duck shared high similarity (99%) with GenBank sequences from C. baileyi obtained from birds (GU816042, GU816039, GU816040, GU816041, and GU816043). C. parvum species isolated from chicks showed 99% sequence similarity with C. parvum sequences from bovine origin (HQ009805 and EF175936). Wang et al. (2014) in China identified 3 genotypes from broiler chickens (C. baileyi, C. meleagridis, and avian genotype II) by PCR and sequencing. In addition, Jasim and Marhoon (2015) recorded C. baileyi in broiler chickens and ducks with 18SrRNA by PCR.

From the current results, it was concluded that the Cryptosporidium spp. infecting ducks according to morphometric and molecular identification might be C. meleagridis.

References


Kuhn, R.C., Rock, C.M. and Oshima, K.H. (2002): Occurrence of Cryptosporidium and Giardia in wild ducks along the Rio Grande River valley in


