Case Report

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An outbreak of severe *Macrorhabdus ornithogaster* infection in common canaries (*Serinus canarius domesticus*), Molecular and pathological assay

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Keywords

Macrorhabdus ornithogaster, proventriculitis, common canary, histopathology

Abstract

Infection by *Macrorhabdus ornithogaster* (MO) formerly called Megabacteriosis has been diagnosed as the cause of depression, wasting, regurgitation, fluffed feathers, lethargy, and soft watery bulky feces and also the cause of mortality in a flock of breeding canaries over a long period of time (3 months) in Mashhad, Iran. Large numbers of *Macrorhabdus ornithogaster* were detected in wet-mount fecal preparations of the feces and direct smears from mucosal scrapings of proventrilculus of one bird under light microscopy. The diagnosis was confirmed by demonstrating a chronic proventriculitis histologically which was associated with MO organisms along with molecular tests.

Abbreviations

MO: *Macrorhabdus Ornithogaster*
Ml: Milli Liter
Mg: Milli Gram
Kg: Kilo Gram
Spp: Species
rDNA: Ribosomal Deoxyribonucleic Acid

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Introduction

Macrorhabdus ornithogaster is defined as an anamorphic Ascomycetes yeast which is grown on the proventriculus and ventriculus junction in birds. It has a wide range of hosts and it can infect many bird species. In the past, it was referred to as 'Megabacterium' which was thought to be a large rod-shaped bacteria until researchers identified this organism as a yeast (Lanzarot et al., 2013).

Due to MO, mycotic proventriculitis is found worldwide and is a well-known chronic fatal disease affecting several species of birds, including budgerigars, canaries, ostriches, chickens, zebra finch and turkeys (Kheirandish and Salehi, 2011).

Whether or not this organism is a bacterium or a fungus has been a controversial issue. In earlier works, this organism was reported to be a bacterium with no nucleus and later it was reported to be lacking characteristics of a fungi (Davis et al., 1981; Van Herck et al., 1984).

MO could be grown in cell culture media adjusted to pH 3-4, containing 20% fetal bovine serum, and 5% glucose under microaerophilic conditions at 42°C (Hanafusa et al. 2007).

Despite the above mentioned studies, more recent works have suggested that MO is a yeast since in vivo trials the organism had shown to be susceptible to amphotericin B treatments, but not to antibacterial agents (Filippich and Perry, 1993). It is also stained strongly positive with calcofluor white M2R and blancophor BA stains binding a polysaccharide (chitin) which is not found in any bacteria. These clues were completed later by finding a nucleus through electron microscopy and in situ hybridization methods (Ravelhofer-Rotheneder et al., 2000).

To the best of our knowledge, MO infection has been previously reported in the budgerigars (Kheirandish and Salehi, 2011) and a recent report of concurrent tuberculosis and macrorhabdosis in a canary (*Serinus canaria*) in Iran (Madani et al., 2015). The aim of the present study is to explain the clinical, pathological and molecular findings of MO infection in canaries.

Case presentation

An outbreak with mortality had occurred in a captive breeding colony with 65 adult pairs and about 125 nestlings in a basement in Mashhad, Iran during 2012 .Clinical signs of depression, wasting, regurgitation, fluffed feathers and lethargy along with soft, watery, bulky feces were detected in more than one-third of the birds with two to three dead birds per week during the previous month and there was also a history of using different Antibiotics such as Enrofloxacin 10 % (1ml/1000ml water 7 days) and Florfenicol (dose of 30 mg/kg body weight) by the owner. Based on the feedback given by the owner, even the birds with good conditions had suddenly become severely depressed and showed ruffled plumage and finally died within 2 to 5 days.

At necropsy, poor body condition was observed in the dead birds and the proventriculus of the affected birds was enlarged and dilated. Grossly, the proventriculus had slightly reddened mucosae. The walls were thickened and the mucosa was covered with grey-white mucus. Petechial haemorrhages and necrotic material were also present.

After gross examination, tissue samples were collected for bacteriologic and parasitological examination to complete the diagnostic and definitive cause of death. Additionally, wet-mount fecal preparations was done in live infected birds and MO was observed under light microscopy.

In order to make definitive diagnosis, intestinal contents of the dead and affected birds were examined for oocysts. Moreover, the eggs of different helminthes along with the liver were analyzed for Salmonella spp. by a specific enrichment method. All bacteriological examinations were performed according to standard procedures.

Specimens taken from proventriculus area which showed gross pathologic changes were fixed in 10% neutral buffered formalin, processed and stained with hematoxylin and eosin (Luna, 1968).

Two sets of the primers were used; the forward primer (AGY1) (GGACTTATATTACTAGTCAGATGG; positions 620–643 in the rDNA of the organism) it did not match with any other reported fungi sequences, which was developed from the 18S rDNA and the reverse primer, Sm2 (CAATACGCCTGCTTTGAACACTC; 761–783).The second set was; the forward primer, Sm1 (ATCTGGTTGATCCTGCCAGTAGTC; positions 2–25) and the reverse primer, Sm2 (CAATACGCCTGCTTTGAACACTC; 761–783), targeting 18S rDNA based on the method used by Tomaszewski et al. 2003 . PCR primer set (AGY1/SM2) was selected as a specific PCR test for MO and PCR primer set (SM1/SM2) was used for sequencing analysis.

The PCR was performed in a TC 512 Temperature Cycling System (Techne, UK) in a reaction volume of 50 µl containing: 25 µl of Taq DNA polymerase 2x master mix red (Ampliqon, Denmark) (containing; 2 mM $MgCl_2$, Tris-HCl pH = 8.5, $(NH4)_2S0_4$, 4 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM dNTPs, 0.2 units/µl ampliqon Taq DNA polymerase inert red dye and stabilizer), 2 µl (10 pmol/µl) of each primer (Bioneer, South Korea) and 25 ng template DNA and double distilled water up to 50 µl final reaction PCR volume. The amplified products were detected by ethidium bromide staining (0.5 µg/ml) after electrophoresis at 100 V for 50 m in 1.5% agarose gels. The PCR product was gel purified using the Invisorb Spin DNA Extraction Kit (Bioneer, South Korea) and was sequenced using the same forward and reverse primers by a commercial DNA sequencing service (Bioneer, South Korea).

Histopathological examinations of all of the affected birds revealed mild to moderate lympho-plasmacytic proventriculitis (Fig. 1). Necrotic debris and exudation containing a large colony of MO were present in proventriculus. Gram-positive MO were present in large numbers in

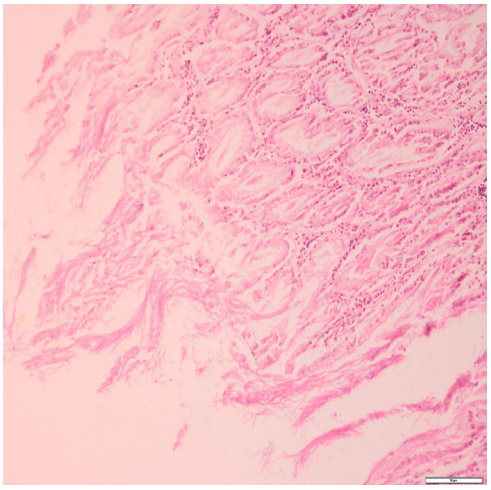

Figure 1
Lympho-plasmacytic proventriculitis associated with necrotic debris in the mucosal surface of the affected proventriculus. Hematoxylin and eosin, 200x.

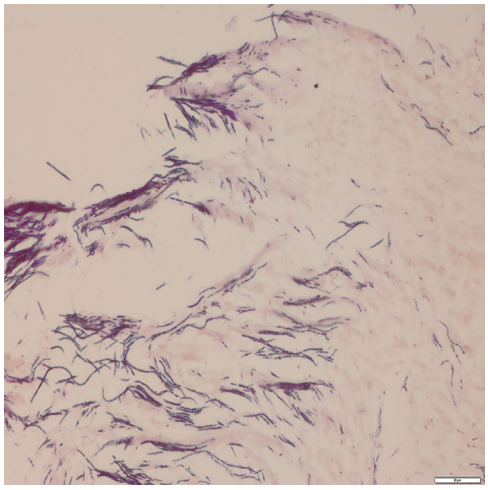

Figure 2
Several Gram-positive rod-shaped organisms (blue-purple) in the mucosal surface of the affected proventriculus infected with Macrorhabdus ornithogaster. Gram's stain, 400x.

the mucus covering the mucosal surface of the superficial proventricular crypts (linearly aligned), and it occasionally invaded the luminal epithelium (Fig.2). Based on clinical history, laboratory findings, and in the absence of lesions in other organs, diagnosis of MO infection was made.

The sequence of the product was compared to the published sequences of MO deposit in GeneBank using BlastN (NCBI, USA) and the Nucleotide sequence was deposited in GenBank under accession number KC433386.

In wet-mount fecal preparations taken from the infected live birds, MO was diagnosed using light microscopy (Fig.3).

The affected live birds were treated using Nystatin (300,000 UI/kg/day PO) based on (Scullion and Scullion, 2004) and also apple vinegar was used at dose of 3 ml in 500 ml to acidify the drinking water which finally relieved the clinical signs.

Discussion

This paper describes clinical, histopathologic and molecular detection of *Macrorhabdus ornithogaster* in an outbreak with high mortality in a flock of common canaries (*Serinus canarius domesticus*). There have been reports of MO infection of captive birds in Europe, North and South America, Japan and Australia (Filippich & Perry, 1993; Martins et al., 2006; Pennycott et al., 1998; Tsai SS et al., 1992). According to its occurrence in bird species kept as pets such as budgerigars, canaries, and finches, it seems to have a worldwide distribution. In mammals, Macrorhabdus-like agents were reported in a dog, a cat and laboratory mice (Cooke, 2000; Rossi, 2000). MO infection has also been reported in Ostriches with clinical manifestations including uneven growth, depression, fasting, leg problems and death. At necropsy, ulceration of gizzard mucosa were noted (Martins, et al., 2006).

Clinical signs of the disease in budgerigars include chronic weight loss, vomiting, dysphagia, diarrhea and finally death (Moore et al., 2001). In canaries, clinical signs are not documented as in budgerigars (Marlier et al., 2006) but they seem to be the same. In sick birds infected by *Macrorhabdus ornithogaster,* there are gross pathologic lesions that include the formation of a thick whitish mucus with dense mats of yeasts covering the whole proventricular mucosal surface. Proventriculitis and ulceration and haemorrhage of the proventriculus mucosa along with depression, debilitation, atrophy of the pectoral muscle and diarrhea are other pathologic findings. The microorganism detection in live birds was carried out through preparation and microscopic evaluation of the wet mounts of the fresh fecal samples or proventricular washes (Lanzarot, et al., 2013). Post mortem diagnosis of MO can be made by histopathologic examination of formalin-fixed specimen taken from the affected areas. In microscopic evaluation, there have been large eosinophilic microorganisms forming log-jam patterns on the surface and also between the mucosal glands (Phalen, 2014).

Many researchers have failed to grow MO on traditional bacterial and fungal media in order to diagnose this organism. However, there is one report regarding the isolation of MO in MRS (de Man, Rogosa and Sharpe) agar media (Gerlach, 2001). The isolation of an MO-like organism from ostriches' proventriculus by means of MRS agar has also been reported(Huchzermeyer et al., 1993) .

Before-death diagnosis of MO infection is typically carried out by a combination of clinical history, physical examination and direct wet mount examination of a crop swab, proventricular lavage and fresh fecal samples, while after-death diagnosis is made by histopathological exam-

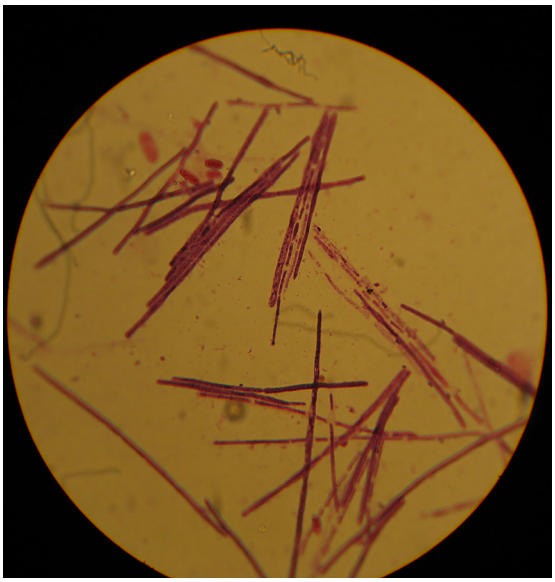

Figure 3
Several microorganisms indicative of Macrorhabdus ornithogaster in fecal samples. Wet mount preparation, ×400.

ination of the proventriculus and ventriculus (Moore, et al., 2001). The causative organism of this disease has not been identified elsewhere in the body of avian species or in the environment (Cooke, 2000; Tomaszewski et al., 2003).

Different antifungal agents could be used to treat MO infection in birds. These drugs include Nystatin, Amphotericin B and other antifungals like Fluconazole. There are also some low toxic antifungal chemicals used to treat MO infection such as Sodium Benzoate and Potassium Benzoate which are not as useful as other mentioned agents in treating this condition (Phalen, 2014).

In the outbreak presented here, we have used several laboratory examinations to rule out other possible causes of death with similar clinical and gross pathologic signs. Some infectious and non-infectious conditions may result in clinical signs similar to MO infection such as trichomoniasis, giardiasis, bacterial and fungal infections of the crop and stomach, helminthe infections of the digestive tract, Bornavirus infection, crop and gastric foreign bodies and heavy metal poisoning. Wet smear was prepared from fresh fecal samples and the organism was observed under light microscope. Bacterial culture was also carried out on liver tissues to rule out salmonellosis. Besides, intestinal contents of both affected and dead birds were examined for the presence of oocyst or intestinal parasites' eggs in which all the samples were negative Molecular detection of MO through PCR on the contents of proventriculus and its lesions tissue in dead birds. Then, accordingly we came to a final diagnosis of MO.

The current study compared to Madani et al. report of an atypical avian tuberculosis with concurrent macrorhabdusis might show us a better viewpoint of MO infection because we have applied different methods to definitively diagnose the organism causing mortality. In addition, we found a severe infection in histopathology and wet smear preparation (Madani, et al., 2015). Since histopathology is a reliable and sensitive method to diagnose MO infection, we have observed typical large, gram-positive organism in proventriculus representative of MO along with inflammation and necrosis while in Madani et al report there has not been any histopathologic diagnosis. According to their report regarding concurrent infection of MO and mycobacteria, it should be noted that the presence of acid-fast microorgansims in the gastrointestinal system of the bird without clinical signs is common.

In this paper, we described clinical, pathologic and molecular characteristics of an outbreak with high mortality in a flock of common canaries caused by severe *Macrorhabdus ornithogaster* infection.

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Abstract (in Persian)

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گزارش شیوع عفونت شدید قناری معمولی با *ماکروورابدوس اورنیتوگاستر*، بررسی مولکولی و پاتولوژیک

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چکیده

عفونت ناشی از *ماکروورابدوس اورنیتوگاستر* که قبلا" مگاباکتریوزیز نامیده می‌شد، بعنوان عامل ایجاد مرگ ومیر به همراه ژولیدگی پرها، بی حالی، لاغری و مدفوع آبکی حجیم در یک گله پرورشی قناری به مدت طولانی (سه ماه) در مشهد ،ایران ، تشخیص داده شد. تعداد بسیار از *ماکروورابدوس اورنیتوگاستر* در گسترش مدفوع و گسترش‌های مستقیم از خراشیدن مخاط پیش‌معده تحت بررسی میکروسکپ نوری مشاهده شد. تشخیص نهایی بر اساس مشاهده آسیب‌شناسی و با استفاده از روش مولکولی PCR بعنوان التهاب پیش‌معده مزمن تایید شد.

واژگان کلیدی: *ماکروورابدوس اورنیتوگاستر*، التهاب پیش معده، هیستوپاتولوژی