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First Detection and Molecular Characterisation of *Eimeria* Spp on Mugilidae Fish *Mugil cephalus* Linnaeus, 1758 in Algerian Coast

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Abstract- The objective of this work is to develop and optimise the ESSP841/CRP999 real-time PCR targeting the 18S ribosomal rRNA of Eimeridae (*Eimeria* spp) found in *Mugil cephalus* from the east coast of Algeria and to determine their pathogenicity and the risk of contamination of consumers. Mugilidae from the east coast of Algeria were captured in search of parasites that could infect them. In the laboratory 816 *Mugil cephalus* were weighed, dissected and eviscerated. In our study we found 378 samples positive for *Eiemeria* sp (46.3%) out of 816 fish samples, i.e. a prevalence of 46.3%, the average intensity was 1.6. The results of the direct examinations are confirmed by real-time PCR using the primer pair ESSP841 CRP999 which was positive for all samples tested. We obtained curves with variable TCs.

Keywords: *mugilidae, mugil cephalus, eimeria spp, RTPCR.*

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First Detection and Molecular Characterisation of *Eimeria* Spp on Mugilidae Fish *Mugil cephalus* Linnaeus, 1758 in Algerian Coast

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Keywords: mugilidae, mugil cephalus, eimeria spp, RT-PCR.

I. Introduction

The genus *Eimeria* comprises obligate intracellular protozoan parasites belonging to the phylum Apicomplexa. Members of this genus cause enteric disease in a wide range of vertebrate hosts, including fish, reptiles, birds and mammals. These parasites complete their development in a single host species and their sporocysts can be recognised by the presence of a Stieda body, an organelle through which the sporozoites exyst. Duszynski and Wilber 1997 several species cause high levels of morbidity and/or mortality in certain hosts, resulting in economic losses in various animal production industries (Daugschies and Najdrowski 2005; Aarthi et al. 2010; Sharma et al. 2018).

A total of 157 species of fish-parasitic *Eimeria* have been described on the basis of sporulating oocyst morphology, host specificity, pathology and geographical distribution (Belova and Krylov 2000). Although these characteristics have traditionally been used to identify *Eimeria* species (Duszynski and Wilber 1997), they are often insufficient for reliable differentiation between species due to overlapping morphometric and biological characteristics (Long et al. 1984; Zhao and Duszynski 2001). A combination of morphological and molecular analyses is therefore necessary to delimit species and determine phylogenetic relationships between them.

The development of molecular tools has allowed not only diagnosis but also the study of genetic variability of pathogens from small quantities of oocysts using molecular markers (Schnitzler et al, 1998; Costa et al, 2001). Fernandez et al (2003) identified species-specific markers for *Eimeria* spp from a cluster of SCAR (Sequence-Characterized Amplified Region) markers. This allowed the use of the polymerase chain reaction (PCR) technique as an efficient and integrated diagnostic method, capable of detecting *Eimeria* species individually or simultaneously in a single reaction (Fernandez et al, 2003; Lien et al, 2007).

Of the forty-two species of Coccidia described in marine fishes (Dykova and Lom 1983), sixteen exist in Mediterranean fishes and are divided into four genera: *Crystallospora* Labbé 1896, *Eimeria* Schneider 1875, *Epieimeria* Dykova and Lom, 1981, and *Goussia* Labbé 1896. Little research has been carried out on these parasites since the end of the last century; the main works are those of Thélohan (1892), Labbé (1896), Léger and Hollande (1922) and finally Lom and Dykova (1981, 1982).

Molecular information on the diversity of *Eimeria* species infecting fish is scarce. Thus, only a few species of *Eimeria* isolated from different marine, estuarine and freshwater fish have been genetically characterised: *Eimeria* percae from perch (*Perca fluviatilis*); *Eimeria anguillae* from the European eel (*Anguilla anguilla*); *Eimeria variabilis*, from the long-billed bullhead (*Taurulus bubalis*); *Eimeria daviesae* on gudgeon (*Gobius fluviatilis*); *Eimeria rutili* on roach (*Rutilus rutilus*); and *Eimeria nemethi* on bleak (*Alburnus alburnus*). (Molnár et al. 2012).

This work constitutes the first study of Coccidia Eimeriidae in the Mugilidae of the Algerian coast. The objective of the present study was to molecularly characterise, at the small subunit ribosomal RNA (rRNA-

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US) locus using the primer pair ESSP841 CRP999, the *Eimeria* isolates obtained from the mullet (*Mugil cephalus*), and to develop a quantitative PCR for rapid detection.

II. Materials and Methods

a) Collection and Processing of Samples

From February 2017 to March 2018 a total of 816 *Mugil cephalus* were caught by fishermen in the east coast of Algeria.

The first step of the present study is to identify positive samples from whole fish samples and examine them under the microscope for oocysts, either by direct methods where samples are examined either freshly, using a 10% concentrated natural formalin buffer, or by staining the samples with iodine or Giemsa to make the internal components clearer.

– The gastrointestinal tract was differentiated into the pyloric cecum and the intestine. The pyloric caeca were homogenised using an Ultra-Turrax® T10 homogeniser (Ika®-Werke GmbH and Co., KG, Staufen, Germany). The intestinal contents were removed by scraping with a scalpel blade and then ground in a mortar with 0.04 M phosphate buffered saline (PBS) pH 7.2. The resulting homogenates were filtered through a set of two sieves (mesh size, 150 and 45 μm) before being subjected to a two-phase concentration of 0.04 M PBS pH 7.2/ diethyl ether (2:1) by centrifugation at 1250xg, 4°C, for 15 min. The supernatants were carefully discarded and the concentration step was repeated until lipid-free sediments were obtained. Finally, the pellets were resuspended in 500-1000 μL of PBS and stored at -20°C.

Aliquots of 10 L of sediment were examined under brightfield microscopy to detect *Eimeria* oocysts (×400 magnification), which were confirmed by appearance, presence of four sporocysts and thin wall. A total of 50 oocysts from several fish specimens were observed under differential interface contrast (DIC) microscopy (×1000 magnification) and measured under a light microscope (AX70 Olympus Optical Co., Ltd., Tokyo, Japan) using a micrometer eyepiece and DP Controller 2.1.1.183 software (©2001-2004 Olympus Optical Co., Ltd.).

b) DNA Extraction

Genomic DNA was extracted from the samples using the Qiamp Stools Quiagen DNA extraction kit.

c) DNA Profile

For the detection of DNA that is extracted from stool samples by using a Nanodrop spectrophotometer (THERMO. USA) for the detection and measurement of the concentration of nuclear acids (DNA and RNA), where the concentration of DNA is detected (ng/μl) and the measurement of the purity of the DNA by reading the absorbance at a wavelength between (280-260 nm) Figure 1.

Wavelength 260 nm: represents the area of maximum absorbance of nucleic acids.

The 280 nm wavelength: is used to establish the ratio and to control the purity of the extraction.

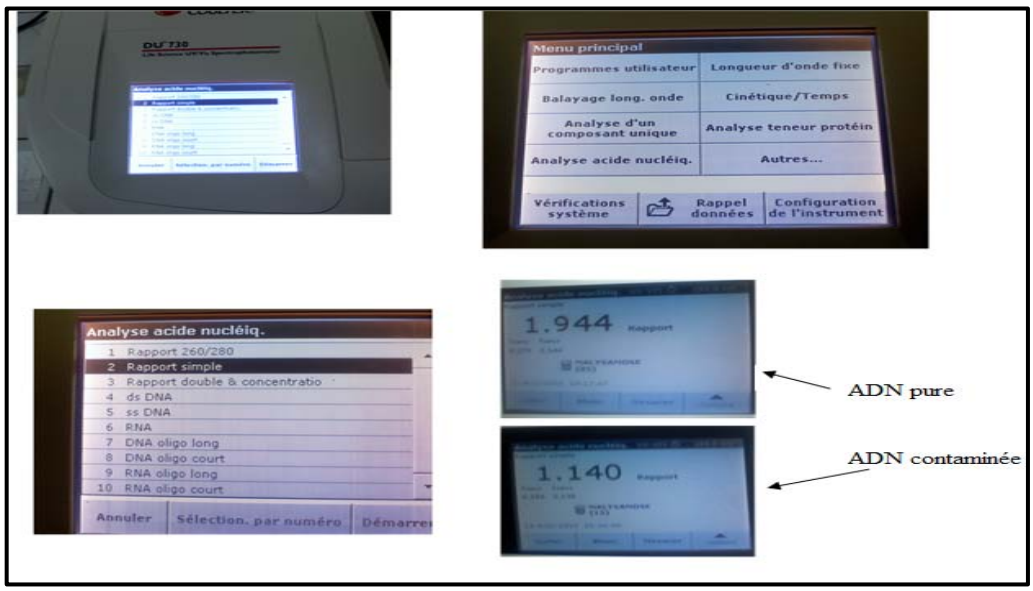


Figure 1: Detection of DNA concentration (ng/μl) and measurement of DNA purity with a Nanodrop spectrophotometer (THERMO. USA)

d) Real-Time PCR Protocols

Real-time PCR performed for the detection of *Eimeria* species from *Mugil cephalus* using primers and TaqMan probe specific to the ITS1 region of the DNA that code for ribosomal RNA. The technique performed as described by Ogedengbe et al. 2011.

e) Real-Time PCR Master Mix Preparation

Real-Time PCR master mix prepared by one-step Reverse Transcription and Real-Time PCR detection kit (Accu Power Rocket Script RT-qPCR Pre Mix, Bioneer. Korea), and done according to company instructions as following Table (1):

Table 1: Explained the main components of the mix for qRT-PCR technique

qRT-PCR Master mix	Volume
2X Green star master mix	25 µL
DNA template	5µL
ITS1 forward primer 10pmol	1µL
ITS1 reverse primer10pmol	1µL
DEPC water	18µL
Total	50µL

Primer

Primers were designed in this study using the complete sequence of the ITS1 region in the rDNA using the NCBI Gene-Bank and Primer 3 plus online and provided by (Bioneer company, Korea) as shown in Table (2):

Table 2: Explained forward and reverse primers used in the qRT - PCR technique with nucleotide sequence and the size of the resulting DNA

Real-Time Primer	Sequence (ESSP841-CRP999)	PCR SIZE
Eimeria spp	5 GTTCTATTTTGTTGGTTTCTAGGACCA-3 5-CGTCTTCAAACCCCCTACTGTCG-3	174 bp

The reaction components of the qRT-PCR mix listed in Table 1 were added to a standard qPCR tube (8-well strip tubes containing Rocket Script Reverse Transcriptase and TaqMan probe pre-mix) (Fig. 2). Next, all strip tubes were vortexed and centrifuged at 3000 rpm for 3 minutes in an Exispin centrifuge and transferred to a real-time PCR thermal cycler.

Figure 2: Preparation of the mix for real-time PCR

f) Real-Time PCR Thermocycler Conditions

Real-Time PCR thermocycler conditions was set up according to primer annealing temperature and RT-qPCR TaqMan kit instructions as following Table (3):

Table 3: Explained Thermal cycler program or qRTPCR technique

Step	Condition	Cycle
Reverse transcriptase	95°C 15 min	1
Pre-Denaturation	95°C 5 min	1
Denaturation	95°C 20 sec	45
Annealing/Extension	60°C 30 sec	
Detection (Scan)		

Thermal cycles were applied to inspect the Real-Time PCR and relying on instructions AccuPower® 2X Green-StarTM qPCR Master Mix as well as by calculating the degree Tm prefixes using the device MiniOpticon Real-Time PCR system BioRad/USA as in Figure (3) below:

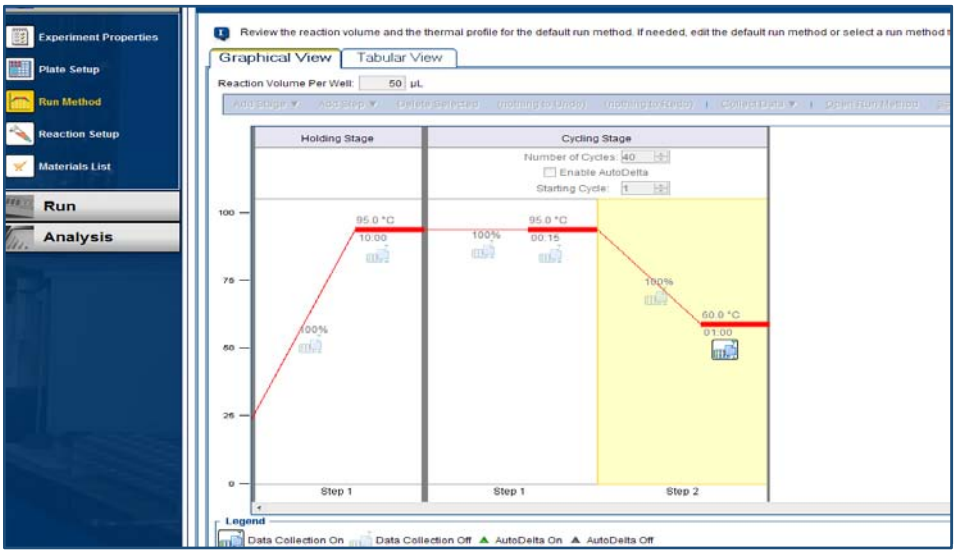


Figure 3: Explained situations of Thermo cycler for REALTIME PCR

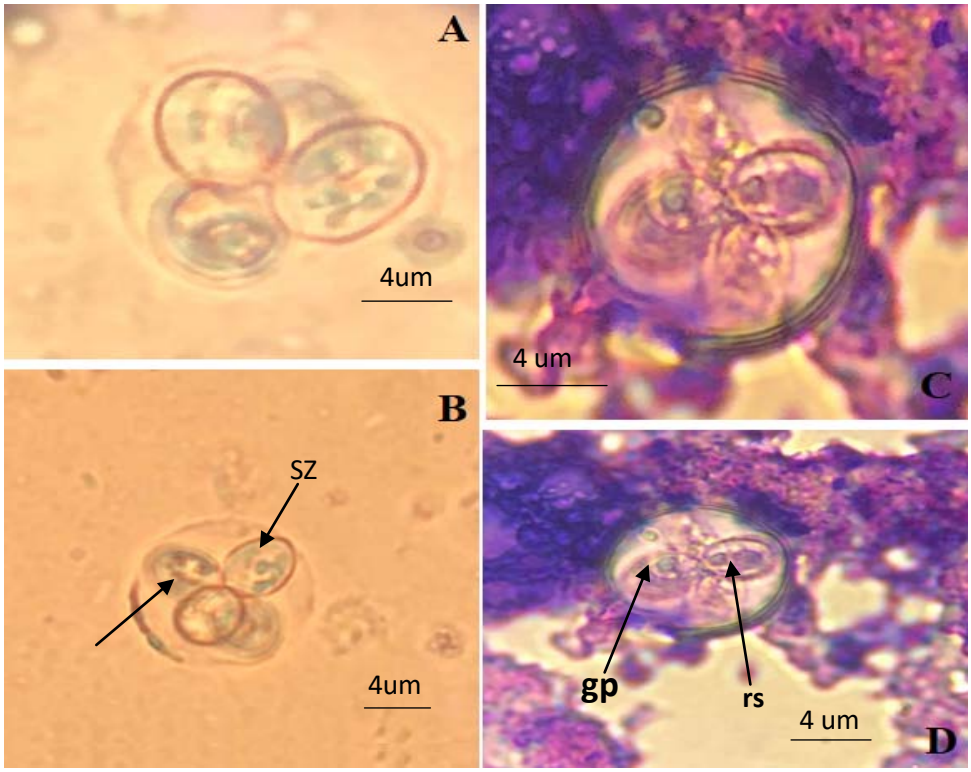
g) Real-Time PCR Data Analysis

qRT-PCR data analysis was performed by calculation the threshold cycle number (CT value) that presented the positive amplification of gene in Real-time cycle number.

III. Results

a) Direct Examination and Staining

In the present study, *Eimeria* oocysts were detected in 378 of 816 (46.3%) gastrointestinal tracts of *Mugil cephalus* examined. This coccidia produces equally spherical oocysts containing sporoblasts and sporocysts (Fig. 4). The oocysts measure 10 ±1.5 um in diameter. The oocyst residue is absent but three polar granules of 2.2 ± 0.5 um diameter each are present (Fig. 4). Each mature oocyst contains four pyriform sporocysts 6.1 ± 0.9 um long and 3.8 ± 0.6 um wide (Fig. 4). At one end of the sporocysts there is a conspicuous projection corresponding to the body of Stieda (Fig. 4). Each sporocyst contains two vermiform sporozoites between which the sporocystic residue is present as three or four refractive granules (Fig. 4).



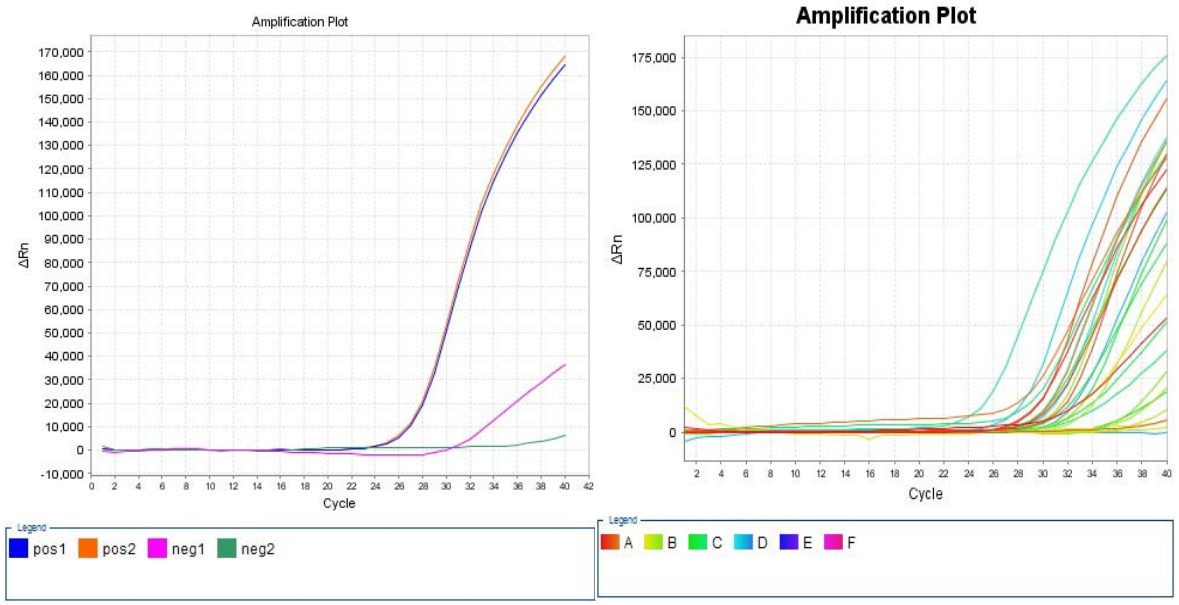
(A) Oocyst containing sporoblasts (bar = 4 um). - (B) Sporulated oocyst. Sporozoites (sz) are visible inside the sporocysts. The arrow indicates the Stieda body (bar = 4 um) - (C) Sporulated oocyst showing three polar granules (gp) and (D) sporocystic residue (rs) in one of the sporocysts (bar = 4 um).

Figure 4: Light microscopy of *Eimeria* sp. oocysts found in *Mugil cephalus*

b) Results of Molecular Examination by qRT-PCR

In the present study, *Eimeria* oocysts were detected in 378 of 816 (46.3%) gastrointestinal tracts of *Mugil cephalus*. Measurements of sporulated oocysts, sporocysts and other morphological characteristics identified the oocysts as *Eimeria* sp. We confirmed by molecular analysis of the small ribosomal RNA subunit (rRNA-SSU) gene, a single sequence of ~174 bp was obtained for all positive samples. The results of the molecular examination using qRT-PCR revealed that of 378 samples collected, 378 (100%) were positive. This complemented and confirmed the results of our microscopic examinations.

The use of qRT-PCR techniques in the specific detection of *Eimeria* sp. showed a fluorescence of the SYBER green dye which was most clearly seen through the formation of an amplification pattern for positive samples from cycle 22 onwards as shown in Figure 5.



A-Positive and negative control profile B-Patient profiles

Figure 5: Amplification graphs of the ITS1 region of *Eimeria* sp. in which the SYBER Green fluorescence represents positive samples above the threshold while control samples are below the threshold

IV. Discussion

The genus *Eimeria* comprises obligate intracellular protozoan parasites belonging to the phylum Apicomplexa. Members of this genus cause enteric disease in a wide range of vertebrate hosts, including fish, reptiles, birds and mammals. A total of 157 species of *Eimeria* that parasitise fish have been described; however, molecular information on these fish parasites is scarce.

In the present study, *Eimeria* oocysts were detected in 378 of 816 (46.3%) gastrointestinal tracts of *Mugil cephalus* in the eastern coast of Algeria. Measurements of sporulated oocysts, sporocysts and other morphological characteristics identified the oocysts as Eimeria sp. By molecular analysis of the small ribosomal RNA subunit gene (rRNA-SSU), by quantitative PCR all direct positive samples came back positive with different Ct's from 22. This confirmed the presence of *Eimeria* sp and complemented the direct examination.

Coccidia of the genus *Eimeria* Schneider, 1875 produce tetrasporidoocysts and dizoicsporocysts. The sporocysts have a Stieda body and sometimes a Stiedasubbody at one end (Lom and Dykovà, 1992). The coccidia described here has these characteristics.

Several studies have used the PCR technique targeting different regions of the *Eimeria* genome, such as the 5S rRNA (the small rRNA subunit (Mushattat and Sukayna (2013), Ogedengbe et al, 2011), the sporozoite antigen gene EASZ240/160 (Qvarnstrom et al., 2005) and the genomic regions ITS-1 (Long and Reid, 1982, Williams, 1998, Lew et al., 2003) and ITS-2 (Lien et al., 2007; Shirley et al., 2005). As the ITS regions are less conserved than the rRNA genes, the wide variation in this region of the DNA sequence between *Eimeria* species makes primer design straightforward and reduces the risk of cross-reactions between different species (Morris and Gasser, 2006). The REAL-TIME test has been shown to be directly comparable in sensitivity and robustness, capable of detecting 10 parasite genomes but not a single one, without being affected by the presence of DNA derived from the host or other species tested (Kirs and Smith, 2007). Each sporulated oocyst contains eight eimerial genomes, suggesting that the DNA equivalent of a single oocyst will be consistently detectable given normal experimental replication (between one and 10 genomes detected per reaction). Mature intracellular stages represent in the order of 10 to 100 eimerial genomes (depending on species and stage (Johnston et al., 2001). This suggests that even a fraction of one can be counted (Damer et al., 2008).

V. Conclusion

This study is the first to characterise *Eimeria* sp in *Mugil cephalus* from the Algerian east coast. Although routine tests such as macroscopic and microscopic diagnosis are important, they are unable to establish a qualitative diagnosis of the *Eimeria* causing the infection in *Mugil cephalus*. The use of molecular methods such as real-time PCR which is characterised by high accuracy, but these methods are expensive compared to routine methods. The use of specific primers for the diagnosis of the ITS1 region is important for the molecular detection of *Eimeria* species that are isolated from the intestines of mules.

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