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The Isolation of Koi Herpesvirus from Ornamental FishImported into The Sultanate of Oman: Implications for Biosecurity

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Abstract: *A health survey was carried out to investigate the prevalence of gill ectoparasites and infectious viral agents in consignments of ornamental fish imported into the Sultanate of Oman. A total of 81 imported goldfish, Carassius auratus, were sampled from three pet shops situated within the Muscat governorate and examined for parasite infection. In addition to these, a further 119ornamental fish from consignments originating from Thailand, Singapore and Taiwan were subjected to virological examination. Ornamental fish species including Carassius auratus (n =83), Cyprinus carpio (n = 5),Siluriformes (n = 4), Piaractus brachypomus (n = 3),Puntigrus tetrazona (n = 4), Hypostomus plecostomus (n = 2), Astronotus ocellatus (n = 4), Trichopodus trichopterus(n = 12) and Hypophthalmichthys molitrix(n = 2) were selected. Pooled samples of spleen, kidney and liver were tested for the presence of Koi Herpesvirus (KHV) and Iridovirus (IV) following standard OIE PCR protocols. The results obtained revealed that 97.53% of the goldfish had parasitic infectionsand 51.31% in Omani native freshwater fish within this, 83.95% had Dactylogyrus (Monogenea), 58.02% had digenean metacercariae, and, 11.11% had Trichodina (Peritricha). Of the pooled fish samples tested for KHV, 2 of the 25 samplestested positive. Sequence analysis of representative PCR products showed 100% genetic homology with the complete genome of cyprinid herpesvirus strain KHV-GZ11 (qi/647842001/KJ627438.1). The virus was detected in asymptomatic goldfish from two pet shops, originally imported from Thailand. The broad host range and pathogenicity of KHV highlights an emerging biosecurity risk. Moreover, new regulations on the importation of live aquatic animals should be studied and established including assessment of health certificate, establishment of quarantine practice and inspection of ornamental fish consignments.*

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I. Introduction

The international movement of live aquatic animals is an important activity for economic, social development and public resource purposes. Since aquaculture is one of fastest and sustainable growing food producing sector, it is considered to be an important vector for international trade of aquatic animals. Since 2007, aquaculture has been engaged in major projects in Oman including abalone and finfish practices. Recently, freshwater fish species, such as carp (*Cyprinus carpio*), freshwater river prawns (*Macrobrachium rosenbergii*) and gilthead seabream (*Sparus aurata*) have been cultured within the country.[1].

Likewise, the culture of ornamental fish by hobbyists has increased. The multimillion ornamental fish industry is characterized by a rich diversity of freshwater and marine species sourced from multiple countries. Worldwide, approximately 1 billion ornamental fish are traded annually, which includes 4,000 freshwater fish species which represent about 90% of the trade. The remainder is comprised of 1,400 marine fish species. Globally, the wholesale of live ornamental fish was estimated by FAO to be 900 million with a retail value of US$ 3 billion.[2].There were approximately 16,000 metric tons of aquatic animals imported into Oman particularly from the United Arab Emirates, Yemen, India, Somalia, Vietnam, France and Indonesia.[3].Approximately nine tons of live ornamental fish entering the country originates from Thailand, Singapore and Taiwan.

An effective biosecurity program with comprehensive procedures, practices and policies should be established to prevent the introduction and spread of aquatic pathogens.[4].Although quarantine is one of the major provisions of the World Organization of Animal Health, OIE (Office International des Epizooties), the Sultanate only depends on the health certificate issued by the country of origin without any quarantine or inspection of fish consignments.[5].

The expanded and occasionally irresponsible global movement of live aquatic animals has sometimes caused serious damage to aquatic food productivity and lead to reduction of ecosystem function. These ecosystem disturbances are underpinned by establishment of exotic viral, bacterial, protozoan and metazoan pathogens from ornamental fishes in farmed and wild fish populations. Thus, demonstrating the presence of specific pathogens in imported ornamental fish has a beneficial importance that could provide a good management practices, prevent

significant losses and even maintain market access. Furthermore, the movement of ornamental fish pose a threat to a country's biosecurity and biodiversity.[6].Commonly encountered species of the gill monogenean*Dactylogyrus* sp. that have been isolated from imported ornamental fish include *D. extensus, D. vastator, D. anchoratus, D. dulikeity* and *D. baueri*.[7,8,9,10].These parasites were found to cause direct losses by mortalities as well as affecting the growth and behavior of fish and subsequently reducing farms production.[11]. Furthermore, the importation of ornamental fish has been shown to be associated with viral diseases outbreaks.[12, 13]. El-Matbouli and Soliman (2011) detected the virus DNA from tissue of asymptomatic goldfish that had been cohabited with KHV infected koi carp by PCR[14]. Therefore, the aim of this study was to assess the prevalence of parasitic and viral agents on a range of imported cyprinid, poeciliid and cichlid fish into the Sultanate of Oman.

II. Materials and methods

Parasitological examinations

Freshwater tropical ornamental fishes were sampled from March to August 2014. Various ornamental fish species were bought from three pet shops in Muscat Governorate including goldfish (*Carassius auratus auratus*), koi carp (*Cyprinus carpio*), catfish (*Ictalurus punctatus*), pacu (*Piaractus brachypomus*), tiger barb (*Puntius tetrazona*), sucker fish (*Hypostomus plecostomus*), oscar (*Astronotus ocellatus*), golden gourami (*Trichogaster trichopterus*), blue gourami (*Trichogaster trichopterus*) and silver carp (*Hypophthalmichthys molitrix*). Apparently clinically healthy native *Garra sp.* (Cyprinidae) freshwater fish (n = 113) were collected from Wadi Daqeq, Lziq village, Sharqiyah governorate. They were subjected to sampling process during the period of June to September, 2014. At the laboratory, both imported goldfish (n=81) and native fishes (n=113) were initially thoroughly examined using dissecting microscope for the presence of any parasites or lesions. Then, wet mount of gills of freshly killed fishes were examined for parasites using a compound light microscope at x100 and x400 magnification which facilitates the visualization of motile parasites. Dehydrated alcohol graded series of parasitic specimens were stained using Mayer's paracarmine stain.

PCR-based detection of KHV

The sample preparation protocol used a salt –based extraction method (DNAzol® reagent) for extraction of KHV DNA. DNA was extracted in 1 ml of DNAzol® reagent and processed according to the OIE's protocol.

Primary PCR was performed using Bercovier TK (Thymidine Kinase) primers which intended to generate 409- bp amplicon corresponding to TK gene (Bercovier et al. 2005). The mixture was subjected to 40 cycles of amplification (95 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min), preceded by an initial denaturing step of 95 °C for 5 min and followed by a final elongation step of 72 °C for 10 min. A template control was included in the PCRs. It also was performed using Gray Sph primers which intended to generate 292-bp amplicon (Yuasa et al. 2005).The mixture was subjected to 40 cycles of amplification (94 °C for 30 sec, 63 °C for 30 sec and 72 °C for 30 sec), preceded by an initial denaturing step of 94°C for 30 sec and followed by a final elongation step of 72 °C for 7 min. A template control was included in the PCRs. Then 3 µl of loading buffer was added in each PCR product in which the product was analyzed by gel electrophoresis with 2% ethidium bromide-stained agarose gel at 100V for 20 min and visualized under UV light.

PCR products were excised from the gel and purified using a commercial kit for gel purification (Geneclean, Q-BIOgene, UK). The purified products were sequenced directly in both directions with the same primers used in the amplification process. Sequence reaction was then analyzed on a genetic analyzer and the alignments and consensus sequences generated using appropriate computer software.

PCR- based detection of IV

DNA separated using spleen and kidney tissue including the spleen cytopathic effect (CPE). The RSIV virion pellet 0.5% in TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0) was added to 1 mg sodium dodecyl sulphate (SDS) and the ml proteinase k 55 °C. After centrifugation at 3000 rpm for 10 min, supernatant fraction were extracted twice with phenol- chloroform in which the DNA precipitated with ethanol and eventually redissolved in 100 µl of TE buffer overnight in Sikkim.

PCR was performed using two protocols in which the former one amplified both RSIV and ISKNV DNA while the other amplified only RSIV DNA. The mixture was subjected to 30 cycles of amplification (94 °C for 30 sec, 58 °C for 60 sec and 72 °C for 60 sec and followed by a final elongation step of 72 °C for 5 min. A template control was included in the PCRs. Then 3 µl of loading buffer wad added in each PCR product in which the product was analyzed by gel electrophoresis with 2% ethidium bromide-stained agarose gel at 100V for 20 min and visualized under UV light.

III. Results

Parasitological findings

The survey revealed that goldfish and native fishes were mainly infected with three different ectoparasite genera which are monogenean, digenean and protozoan ciliates. The most dominant parasites in goldfish were dactylogyrid monogeneans (Table 1) (83.95%). The second more abundant parasite was digenean metacercaria (58.02%). The lowest prevalence of parasitic infection was recorded with protozoan, Trichodina sp. (11.11%). Out of the total 113 native fishes, the number of infected fishes was 58 (51.31%). The highest prevalence was recorded with digenean metacercarial infection (36.28%), followed by Dactylogyrus spp. (31.86%). On the other hand, Gyrodactylus sp. was detected in 7.08% of native fishes.

Virological findings

PCR-based detection of KHV

Specific PCR products corresponding to 292 bp fragment were amplified using the modified Gray Sph primer set. Interestingly, PCR results of the DNA extracted from commercially imported fish tissues revealed the presence of specific fragment 292 bp of KHV in kidney, spleen and liver tissues of goldfish. Out of 25 pooled samples of different imported fish species tissues, 2 pooled samples were positive in which the prevalence was recorded to be 8 %. Of which the two pooled samples were sampled from pet shops. Nevertheless, the PCR using TK primer set did not result in the amplification of a product. Apart from that, the PCR result using TK and modified Gray Sph primer sets for the samples of *Garra sp.*did not result in amplification of a product.

PCR-based detection of RSIV

The PCR result for the tissues of the samples of different imported ornamental fishes using the 1F/1R and 4F/4R primer sets not revealed any amplification of a product. Furthermore, the PCR result using 1F/1R and 4F/4R primer sets for the samples of *Garra sp.*did not result in amplification of a product. Nevertheless, both the negative and positive controls performed consistently and as expected.

Sequencing analysis results

Two PCR products derived from goldfish spleen, kidney and liver tissues were subjected to sequencing. Both PCR products were identical in sequence. The analysis of the 292bp sequence obtained revealed that it shared 100% genetic homology with the complete genome of CyHV-3 of the strain KHV-GZ11 (Gene bank accession number: KJ627438.1). The results indicated that the CyHV-3 detected in the goldfish from pet shops grouped with previously identified distinct fatal European genotype of CyHV-3.

IV. Discussion

This is the first report on the parasitic fauna of tropical ornamental fishes imported into Oman. In the current investigation, the overall parasitic infection in goldfish is 97.53% by at least one parasite species. This infection was dominated by monogeneans belonging to the genus*Dactylogyrus* followed by digenean metacercaria and then *Trichodina sp.* The result of this study seemed to reaffirm the findings of previous studies conducted by many researchers.[8, 10, 15]. These studies reported total parasitic infection in freshwater goldfish as 61.43%, 63.33% and 75%. The previous studies also confirmed the presence of *Dactylogyrus sp.*, digenean metacercarial stage and *Trichodina sp.* Another study on the diversity and distribution of external parasites from freshwater fishes conducted in Thailand indicated that monogenean was the dominant group of ectoparasites. Of which *Trichodina sp.* and *Dactylogyrus sp.* was widely distributed and had the highest number of species respectively.[16]. The current finding support the fact that the obligatory ectoparasite, *Trichodina* is more prevalent in high stocking densities with high nitrite, nitrate and phosphorus concentration that facilitate its mode of reproduction, binary fission.[17]. On the other hand, fish were highly parasitized with monogenean parasites namely *Dactylogyrus* which are the most common parasite in freshwater fish. The highly prevalence and wide spread of this parasite may due to a number of reasons including their easy transmission under unfavorable management conditions coupled with their short, direct life-cycle and high reproductive rate. Nevertheless, the overall prevalence was considered high in the imported goldfish as well as in the native fish with minor differences in the type of detected parasite which may related to seasonality of the occurrence and water temperature.

The Koi herpesvirus is often spread through the international trade of live apparently healthy fish species including goldfish species. Unlike other viral disease, the isolation of CyHV-3 on cell culture is unreliable and inapplicable test as it is difficult to be detected in subclinical or asymptomatic cases. Several PCR tests have incorporated in the detection of CyHV-3 including real-time PCR assay, loop- mediated isothermal amplification or real-time PCR.[18, 19]. This related to the specificity of each primer used and the ability of each method to detect the target DNA amplicons of a particular size. In the current study, the amplified product exhibited 100% genetic homology with CyHV-3 strain KHV-GZ11complete sequence (accession numberKJ627438.1). Thus, the recent study confirmed the presence of Cy-HV-3 in 8% of apparently healthy imported fish species in particular goldfish

species. This implied that the CyHV-3 replicated in healthy–looking goldfish tissue, the potential carrier of the virus. This finding is in accordance with the study which demonstrated that the goldfish is reservoir for the CyHV-3[14], therefore allowing the dissemination and transferring of the virus to carp in favorable conditions and with the presence of stress factors. In CyHV-3 infected goldfish, there were neither clinical signs nor deaths. This may because several goldfish defense proteins that interact with CyHV-3 such as beta-2-microglobin, complement factor B/C2A, cathepsin Z and Myofibril- bound serine proteinases which suppress cell division in infected cells and thus the virus is not permitted to replicate and the KHVD effects will be suppressed.[20].

V. Conclusions

The importation of subclinically infected goldfish with the highly contagious CyHV-3 disease as well as variety of ectoparasitic monogeneans, digenean and protozoan threatens the country's biosecurity. The risk is increased with the lack of pre and post border control measures including authorized health certificates, advanced inspection tests and quarantine detention. Moreover, the lack of information on the pathogenic viruses and parasites of imported ornamental fishes, the role of carrier or reservoir species on the transmission of exotic pathogens and thus the establishments of exotic pathogens in native fish can exacerbate the situation. Effective approach of risk analysis through identification and assessment of fish pathogens recorded in live ornamental fish should be applied.

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Table1 Prevalence of parasites recovered from goldfish and from an Omani population of *Garra* sp.

Parasites	Host species	Prevalence (%)
***Dactylogyrus* sp.**	Goldfish	83.95
	Garra sp.	31.86
Digenean metacercaria	Goldfish	58.02
	Garra sp.	36.28
***Trichodina* sp.**	Goldfish	11.11
***Gyrodactylus* sp.**	*Garra* sp.	7.08

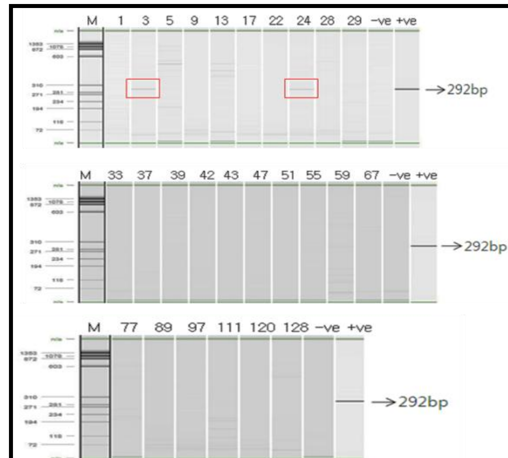

Figure 1 Agarose gel electrophoresis (2%) demonstrates amplification using modified GraySph primers (292 bp) for the CyHV-3 by direct PCR test*.
*Lane M: 100 bp DNA ladder; Lane 1-128: DNA extracted from tissues of 25 pooled samples of imported fish species; lane 128: DNA extracted from tissues of sample of freshwater naïve fish; Lane –ve: negative extraction control; Lane +ve: Positive control.

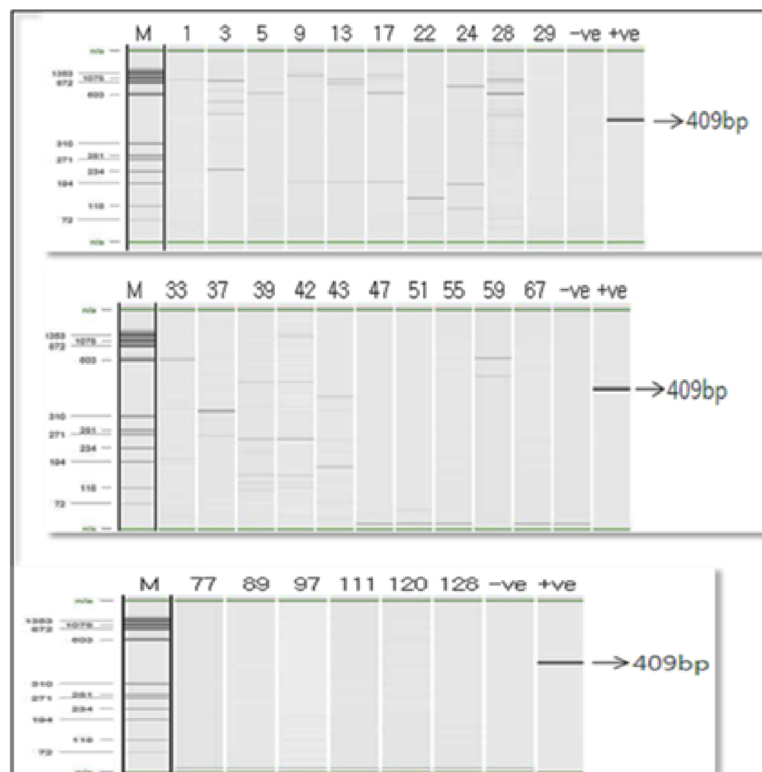

Figure 2 Agarose gel electrophoresis (2%) demonstrates amplification of the TK gene of the CyHV-3 by direct PCR test*.
*Lane M: 100 bp DNA ladder; Lane 1-127: DNA extracted from tissues of 25 pooled samples of imported fish species (spleen, kidney & liver); lane 128: DNA extracted from tissues of sample of freshwater native fish; Lane –ve: negative extraction control; Lane +ve: Positive control.

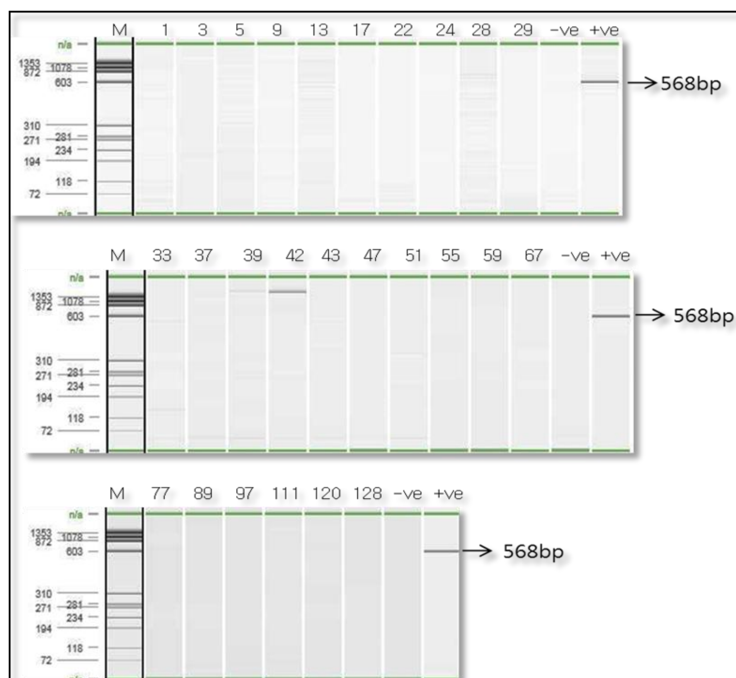

Figure 3 Agarose gel electrophoresis (2%) demonstrates amplification of RSIV and ISKNV DNA (570-bp) by direct PCR test*. *Lane M: 100 bp DNA ladder; Lane 1-128: DNA extracted from tissues of 25 pooled samples of imported fish species (spleen, kidney & liver); lane 128: DNA extracted from tissues of sample of freshwater naïve fish; Lane –ve: negative extraction control; Lane +ve: Positive control.

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