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Parasites of zoonotic interest in selected edible freshwater fish imported to Australia

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ABSTRACT

Australia imports a significant amount of edible freshwater fish. The safety of the imported product is therefore of great importance. Previous research has shown that certain types of edible freshwater fish imported into Australia are not compliant with Australian importation guidelines and additionally are contaminated with many species of parasites, some of which may cause illness in humans if consumed. The present study, to the best of authors knowledge, is the first to publish the occurrence of zoonotic parasites in edible fish imported into Australia. Eustrongylides sp. Jägerskiöld, 1909 (P. 15.5%), family Dioctophymidae; Euclinostomum sp. Travassos, 1928 (P. 4.8%), family Clinostomidae, were recovered from imported edible and consumer ready Channidae fish and Isoparorchis sp. Southwell, 1913 (P. 11%), family Isoparorchiidae, from imported edible Bagridae fish. Euclinostomum sp. and Isoparorchis sp. were identified using morphological method. Molecular identification of Eustrongylides sp. was achieved through sequencing of the 18S ribosomal RNA gene sequence. Eustrongylides sp. and Isoparorchis sp. have been identified as the causative agent in cases of human infection and are a recognised zoonosis. Euclinostomum sp. is considered to have zoonotic potential and for this reason this species has been included in the importation risk assessments for freshwater fish from certain countries. This study confirmed the presence of zoonotic parasite species in edible imported fish. Whilst this fish product was frozen and parasites therefore inactivated, both fish species according to importation commodity codes, at the time this manuscript was written, are permitted entry into Australia chilled. Further study using a greater sample size is required to understand the human health risks.

1. Introduction

In 2019, 3873 t of freshwater edible catfish alone was imported into Australia (FRDC, 2019). Parasite contaminated fish, when combined with safety weaknesses in the global supply chain, is a potential human health risk (Fig. 1). As a result, there have been outbreaks of human illness resulting from the importation of edible seafood reported globally (Attia et al., 2012; Autier et al., 2019; Cole et al., 2014; Ko et al., 2019; Paugam et al., 2009; Shimizu et al., 2012; Wicht et al., 2007; Yera et al., 2013; Youssef et al., 1989) and it is possible that many more cases are unreported. In Australia, the true nature of human illness from parasites in imported edible fish has yet to be adequately investigated.

To provide justification for examination of imported freshwater fish products, Williams et al. (2021) developed a risk scoring

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system to identify countries which may be high risk for supply chain breaches which may then impact the safety of the fishery product imported into Australia. The authors used six predictor variables to score the outcome variable of 'Freshwater fish high risk' and in the same study allocated each 'Country' with a unique numerical and anonymous identifier. The Williams et al. (2021) study identified a number of supply chain breaches which were inconsistent with Australian importation policy for the respective examined fishery products. Identified breaches included retained livers/intestine/gills, serious physical hazards, mud and other varied types of debris. In addition, in the same study, 200 parasites in total were recovered from two species of edible fish (N = 121) imported into Australia from high-risk Country 22 (22: anonymous numerical country identifier).

Zoonotic parasites in edible fish imported to Australia are a human health concern as the infective stage of many zoonotic fish borne parasites have been demonstrated as highly resilient to varied methods of storage and preparation. For example, Fan (1998) demonstrated *Clonorchis sinensis* metacercaria frozen at $-12\,^{\circ}$ C for 10-20 days and $-20\,^{\circ}$ C for 3-7 days, or treated with a heavy salt concentration (fish/salt = $10\,$ g/3 g) remained viable and produced infection in rats. Whilst it is unknown if metacercariae of other seafood borne zoonotic species are equally resilient it is clear that freezing, storing infected freshwater fish in heavy salt or brine, and certain types of cooking methods may not be effective in negating their infectious potential. Similarly, *Anisakis* larvae at high salinity for three weeks showed no motility however after culture 13/71 larvae identified became motile (Grabda and Bier, 1988). Nematode larvae have been demonstrated to survive freezing at $-5\,^{\circ}$ C to $-10\,^{\circ}$ C (Wharton and Aalders, 2002) and Sánchez-Alonso et al. (2018) demonstrated the survival rate ranged from 48 to 63% after freezing at $-10\,^{\circ}$ C.

Fish borne zoonotic parasites if consumed in undercooked fish may cause a variety of clinical manifestations in humans, from mild and transient to chronic, severe or at times life threatening (Bao et al., 2017; Limsrivilai et al., 2014; Piscaglia et al., 2014; Sadaow et al., 2017). According to Shamsi and Sheorey (2018), a lack of diagnostic suspicion in Australia may contribute to the low number of reported cases of human infection from fish borne parasites. In a survey of Australian doctors, <10% were able to name one zoonotic seafood borne parasite and only 3.8% believed that any risk existed (Seal et al., 2020). By inference in Australia the diagnosis of human illness from unfamiliar zoonotic parasites in imported fish may be very difficult. Therefore, the interface between imported fish and Australian inspection procedures for zoonotic parasites should be strong.

According to the latest Australian 'Imported Food Control Order' (Aust. Gov., 2020), no additional tests have been advised for detection of zoonotic parasites in edible seafood on entry to Australia. The procedure for imported edible seafood may involve label inspection (FSANZ, 2019), visual inspection (Fig. 2) or under Subsection F (25; 1–2) of the 'Imported Food Control Regulations 2019', (Aust. Gov., 2019) could be sent for analysis to determine if the imported product is a risk to human health or complies with an applicable food safety 'standard.' Of the schedules in the 'Australian and New Zealand Food Standards Code (the 'standard') for imported food none are specific to parasite contamination (FSANZ, 2021a) and in the current the FSANZ advice on imported food no category is provided for parasites in seafood (FSANZ, 2021b). Visual inspection is likely to detect only macroscopic parasite lesions and as a tool seems inadequate to detect microscopic parasites (Shamsi and Suthar, 2016a). Therefore, the aim of this study was to identify parasites collected from imported edible freshwater fish to the lowest taxonomic unit possible using morphological methodology and

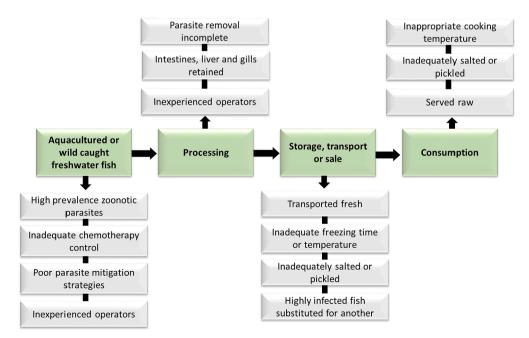


Fig. 1. The global fish supply chain and risk points for zoonotic parasites in edible fish. In green are the basic steps, from farm/capture to consumption which edible imported freshwater fish may follow. In grey are the risk factors at each stage of the supply chain which may increase the risk of zoonotic parasites in edible freshwater fish. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

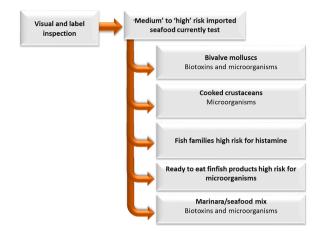


Fig. 2. Current inspection procedure for edible seafood imported into Australia.

to confirm the identity of damaged larval nematodes or those with poor morphology using molecular methods. The associated human health risks of identified zoonotic or potentially zoonotic parasite species will be discussed.

2. Materials and methods

2.1. Parasite collection

Two hundred parasites in total from two species of edible imported Channidae (Sp. A) (n=103) and Bagridae fish (Sp. B) (n=18) were collected in Williams et al. (2021), for identification in the present study. Both Sp. A and Sp. B were imported into Australia from Country 22 (22: anonymous numerical country identifier). Table 1 provides fish details. The methods used for parasite isolation followed Shamsi and Suthar (2016b) for whole fish and artificial digestion of all fish followed the method described in Bier et al. (2001). Collected parasites were stored in 1.5 mL sterile Eppendorf® tubes containing 70% ethanol pending morphological/and or molecular identification in the present study. The present manuscript follows Williams et al. (2021) "any information which may lead to the identification of an included country has been omitted from the manuscript, auxiliary tables and figures. This information includes country descriptions, fish species names, or citations."

2.2. Parasite preparation

Preparation of parasites for morphological examination were according to each morphotype and followed methods described in Table 2. For molecular study of nematodes a small piece was excised from the mid-body of a representative specimen of each larval nematode as described in Shamsi et al. (2008). The anterior and posterior portion of nematode specimens were slide mounted and cleared with lactophenol. Digenean specimens were removed from 70% ethanol and rehydrated with a 50% ethanol and distilled water series before staining with Semichon's acetocarmine. Specimens were then dehydrated with a graded ethanol series (50%, 70%, 80%, 90%, 95% (twice), absolute (twice)), and cleared with xylene based on the method in Sohn and Na (2018). Cycling time for re/and dehydration was adjusted according to specimen size. Specimens were slide mounted with Canada Balsam.

2.3. Morphological identification

Selected specimens were studied morphologically and characteristics of importance, following publications in Table 2, were

Table 1 Details of fish in the present study.

Fish ID	Number of fish	Country of origin	Packaging and fish ~length	Fish details
Channidae sp. (Sp. A)	103	22	Consumer ready but a wide variation of processing standards. Many partially eviscerated and with gills still remaining. Fish frozen in single layer and surrounded with ice. Fish ranged between 9 and 12 cm in length.	Primarily freshwater aquaculture or polyculture. Considered voracious, predatory carnivore of small fish and also feeds on worms and insects. Habitat includes stagnant or muddy aquatic environments
Bagridae sp. (Sp. B)	18	22	Non-consumer ready. Fish uneviscerated with head and gills present. Fish frozen in single layer and surrounded with ice. Fish were generally uniform in size (\sim 6 cm in length).	Freshwater commercial species which feeds on crustacea, insects, or plant matter. Habitat includes freshwater lowland basins/rivers.

Table 2 Sources used to identify parasites in this study.

	<u> </u>
Genus or species	Reference used for morphological characteristics description
Euclinostomum sp.	Caffara et al. (2016)
	Jhansilakshmibai and Madhavi (1997)
	Vankara et al. (2011)
Eustrongylides sp.	Abro et al. (2020)
	Gupta (2019)
	Mazzone et al. (2019)
Isoparorchis sp.	Shimazu et al. (2014)
	Sohn and Na (2018)
	Vankara et al. (2011)

measured using an eyepiece micrometre (BX-43 Olympus Microscope, Olympus Corporation, Japan). All measurements are given in millimetres, unless stated otherwise. The range of measurements are given in the format of length x width mm or specified as length or width only. Drawings were made using BX-43 Olympus Microscope, Olympus Corporation, Japan fitted with a drawing tube. Image capture of specimens was conducted using an Upright Motorized Microscope ECLIPSE Ni-E, Nikon, Japan. Morphological description is provided for *Isoparorchis* sp. and *Euclinostomum* sp. Due to the poor quality of larval *Eustrongylides* specimens, molecular method only was used for identification.

2.4. Genetic characterisation

Genomic DNA from *Eustrongylides* was extracted according to Shamsi et al. (2019) using DNeasy Blood & Tissue Kits (QIAGEN, Germany) and eluted by 40 μl of elution buffer. The 18S genes have been successfully used as a genetic marker for sequencing *Eustrongylides* sp. Therefore, in the present study PCR reaction (25 μl) was conducted to amplify the 18S ribosomal RNA gene region of ten *Eustrongylides* larval nematodes. Primer sets used for *Eustrongylides* were Forward: SOBO18S (5'-TTTGGTTTTCGGATCTGAGG-3') and Reverse: SOBO18S (5'-GTACAAAGGGCAGGACGTA-3'). Initial cycling time was 95 °C for 2 min, followed by 95 °C for 30 s, 50 °C for 40 s, 72 °C for 45 s, 40 cycles, followed by a final extension at 72 °C for 10 min.

For each amplicon, a 3 μ l aliquot was examined on a 1.5% w/v agarose gel which was stained with GelRedTM after which a photograph was taken using a gel documentation system. Nine samples of *Eustrongylides* larval types were sent for sequencing to the Australian Genome Research Facility (AGRF). The samples sent for sequencing were prepared using identical primer sets as for the PCR. The chromatogram and sequence data were observed using Sequence Scanner software (Applied Biosystems® Genetic Analysers). BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to conduct homology searches. MUSCLE (in MEGA v. 10) (Kumar et al., 2018; Kumar et al., 2016) was used to align sequences.

2.5. Parasite examination and calculations

The prevalence (P), mean intensity (MI), and mean abundance (MA) of the parasites described in this paper were calculated following Bush et al. (1997)

 $P = (number\ of\ infected\ fish/total\ number\ of\ examined\ fish) \times 100;$

MI = (number of parasites/number of infected hosts);

Table 3List and number of zoonotic parasites identified from imported fish Species A & B.

Fish and number (n =)	Parasite species	No. of fish infected	Range in infected fish	Prevalence (%)	Total no. of parasites found	Mean intensity	Mean abundance	GenBank accession numbers this study	GenBank accession number match
Channidae Sp. A (n = 103)	Eustrongylides sp.	16	0–2	15.5	20.0	1.20	0.20	OK104103; OK104104; OK104105; OK104106; OK104107; OK104108; OK104109; OK104110	AB558484
	Euclinostomum sp.	5	0–5	04.8	11.0	2.20	0.10		
Bagridae Sp. B (n = 18)	Isoparorchis sp.	2	0–3	11.0	04.0	2.00	0.22		

MA = number of parasites/total number of examined hosts.

3. Results

3.1. Prevalence

Of the 200 parasites collected in Williams et al. (2021) from 103 imported, edible and consumer ready of Sp. A (fish eviscerated) and from 18 imported, non-consumer ready edible fish of Sp. B (fish uneviscerated) (Table 3), a total of 35 parasites were identified in the present study as zoonotic or potentially zoonotic. Twenty *Eustrongylides* sp. (P, 15.5%; MI, 1.20) and eleven *Euclinostomum* sp. (P, 04.8%; MI, 2.2) were recovered from Sp. A. Four *Isoparorchis* sp. were recovered from Sp. B (P, 11.0%; MI, 2.0). *Eustrongylides* sp. (Fig. 3A & B) were encysted in the mesentery of the intestine in clear membranes and one only was found encysted in the musculature of the abdominal wall. All were larval stage. Of the ten encysted *Euclinostomum* sp. nine were found in or on the liver and one was identified after artificial digestion of musculature. One ex-cysted metacercariae (Fig. 3C) was found free in the abdominal cavity. *Isoparorchis* sp. were recovered from the abdominal cavity of non-consumer ready fish and all were ex-cysted larval metacercariae stage (Fig. 3D-F). Table 4 contains a list of the parasites previously reported in Sp. A and Sp. B.

3.2. Morphological identification of parasites

3.2.1. Parasites identified in edible consumer ready fish, Sp. A

Phylum Platyhelminthes.

Class Trematoda.

Family Clinostomidae Lühe, 1901.

Euclinostomum sp. Travassos, 1928.

One ex-cysted specimen in the present study corresponds with the measurements in Jhansilakshmibai and Madhavi (1997) for a 55-day old *Euclinostomum heterostomum* metacercariae. The body is 6.50 mm long, 1.75 mm wide at the ventral sucker and 2.62 mm wide at the anterior testes. The body is cylindrical with a rounded posterior and truncated at the anterior end. The body tegument is smooth although small sections are damaged. The ventral sucker is large $(0.92 \times 1.12 \text{ mm})$, surrounded by a muscular acetabulum situated in the anterior third of the body. The caecum bifurcates horizontally at the pharynx and joins two main lateral intestinal caeca canals with irregular and blind diverticular (11–12 branches). There are two testes. The anterior testes are U shaped (right arm 0.25 mm and left arm 0.30 mm long) and 0.62 mm at the widest point, 0.52 mm at the branch junction and 0.20 mm at the base and occupy the posterior end of the anterior half of the body. The posterior testes are V shaped and situated in the posterior third of the body directly below the anterior testes. The right arm of the testes is 0.50 mm in length, the left arm 0.42 mm and is 0.50–0.20 mm wide. The ovary is round $(0.15 \times 0.15 \text{ mm})$ and granular, indistinct around external edge and contacts the anterior testis along the testes posterior edge. Vitellaria are follicular but not well defined along the left margin of the specimen and run laterally along the body margin from the posterior ventral sucker to posterior margin.

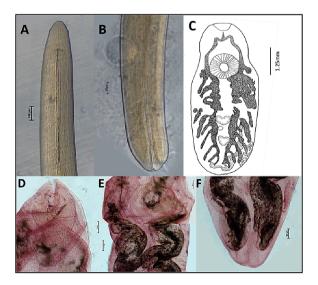


Fig. 3. Eustrongylides sp., Euclinostomum sp. and Isoparorchis sp. 3A. anterior and 3B. posterior of Eustrongylides larvae. 3C. Euclinostomum ex-cysted metacercariae. 3D Isoparorchis sp. metacercariae anterior, E. mid-section with muscularised acetabulum surrounding ventral sucker and F the posterior. Characteristic undulating intestinal caecum morphology in view E and F.

Table 4Previous reports of parasites from fish of Sp. A and Sp. B from Country 22 and nearby regions.

Hosts	Parasite taxa	Family	Site	Localities	Reference
Channa sp.	Allocreadium handiai	Allocreadiidae	Intestine	Dhaka, Sylhet, Mymensingh, Bangladesh	Coil and Kuntz (1960), Khalil et al.
(Snakehead					(2014) and Farzana et al. (2019)
fish)	Anchistrocephalus sp. (unlikely)	Triaenophoridae	Intestine, liver	Chittagong, Bangladesh	Ali (1968)
	Ascaridia sp. (uncertain)	Compositae	Digestive tract, viscera, body cavity	Dhaka, Bangladesh	Huq et al. (1985)
	Ascaridia sp.	Compositae	Intestine	Mymensingh, Bangladesh	Farzana et al. (2019)
	Ascaris sp.	Ascarididae	Stomach	Dhaka, Bangladesh	Huq et al. (1985)
	Asymphylodora indica (uncertain)	Lissorchiidae	Intestine	Dhaka, Bangladesh	Coil and Kuntz (1960)
	Camallanus intestinalus (not valid species. Cannot find any synonym)	Camallanidae	Intestine	Dhaka and Sylhet, Bangladesh	Bashirullah (1974) and Khalil et al. (2014)
	Contracaecum sp. larva	Anisakidae	Intestine	Mymensingh, Bangladesh	Ali (1968) and Farzana et al. (2019)
	E. heterostomum	Clinostomidae	Liver	Dhaka, Bangladesh	Arthur and Ahmed (2002)
	Euclinostomum sp. and E. heterostomum	Clinostomidae	Stomach, intestine	Mymensingh, Bangladesh	Farzana et al. (2019)
	E. heterostomum	Clinostomidae	Liver, kidney, peritoneum, muscle, and ovary	Local fish market, Aligarh, North India	Shareef and Abidi, 2015
	E. heterostomum	Clinostomidae	Coelomic cavity & liver	River Godavari, Rajahmundry, India	Vankara et al., 2011
	E. multicaecum	Clinostomidae	Stomach and intestine	Sylhet and Mymensingh, Bangladesh	Khalil et al. (2014) and Farzana et al. (2019)
	Eucreadium daccai	Opecoelidae	Intestine	Dhaka, Bangladesh	Bashirullah (1972)
	Genarchopsis avitellarium	Derogenidae	Stomach	Assam, India	Varma (1983)
	G. bangladensis (syn. G. goppo)	Derogenidae	Stomach	Tongi Hatchery, Gazipur & sewage	Alam et al. (2010)
				treatment lagoon, Narayangonj, Bangladesh	
	G. dasus (syn. G. goppo)	Derogenidae	Intestine, stomach, liver, intestine & body cavity	Mymensingh, Bangladesh	Chandra et al. (2011)
	G. dasus (syn. G. goppo)	Derogenidae	Liver, stomach	Sylhet, Bangladesh	Khalil et al. (2014)
	G. дорро	Derogenidae	Stomach and intestine	Mymensingh, Bangladesh	Farzana et al. (2019)
	G. macrocotyle	Derogenidae	Small intestine	Dhaka, Bangladesh	Coil and Kuntz (1960)
	G. ozakii (syn. G. goppo)	Derogenidae	Stomach and intestine	Dhaka, Bangladesh	Bashirullah (1972)
	I. hypselobagri (uncertain species classification)	Isoparorchiidae	-	Jaipur, India	Mahajan et al. (1979)
	I. hypselobagri (uncertain species classification)	Isoparorchiidae	Liver, stomach	Sylhet, Bangladesh	Khalil et al. (2014)
	Lernaea cyprinacea	Lernaeidae	Skin, under accessory respiratory organs, above gill clefts, liver, abdominal muscles	Dhaka, Bangladesh	Hossain et al. (1980)
	Neocamallanus bareilliensis	_	Intestine	Bareilly (U. P.) India	Sharma and Sharma (1980)
	N. ophicephali	_	Pyloric caeca, intestine	_	Ahmed (1981) and Bashirullah (1973)
	N. saharanpurensis	Opecoelidae	Stomach, intestine	Dhaka, Bangladesh	Bashirullah (1972)
	Pallisentis (Brevitritospinus) allahabadii	Quadrigyridae	Intestine, liver, mesenteries	Barisal, Dhaka, Bangladesh	Ahmed and Rouf (1981) and Ahmed (1981)
	Pallisentis (Pallisentis) nagpurensis	Quadrigyridae	Intestine	Barisal, Dhaka, Bangladesh	Bashirullah (1973)
	Pallisentis ophiocephali (syn. Pallisentis (Demidueterospinus) ophiocephali)	Quadrigyridae	Intestine, viscera and stomach	Dhaka, Sylhet, Mymensingh, Bangladesh	

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Table 4 (continued)

Hosts	Parasite taxa	Family	Site	Localities	Reference
					Huq et al. (1985), Chandra (1985), Khalil et al. (2014) and Farzana et al. (2019)
	Porrocaecum sp.	Ascarididae	Intestine	Mymensingh, Bangladesh	Farzana et al. (2019)
	Phyllodistomum chauhani	Gorgoderidae	Intestine or body cavity	Dhaka, Bangladesh	Chandra and Banerjee (1993)
	Procamallanus sp.	-	Intestine	Dhaka, Bangladesh	Huq et al. (1985)
	Senga ophiocephalina	Bothriocephalidae	Intestine	Mymensingh, Bangladesh	Farzana et al. (2019)
	Trypanosoma punctati (punctati not valid species)	Trypanosomatidae	Blood	Kerala, India	Shanavas (1991)
	Trypanosoma saulii n. sp. (saulii not valid species)	Trypanosomatidae	Blood	Bareilly, India	Gupta et al. (2006)
Mystus sp.	Allocreadium handiai	Allocreadiidae	Intestine	Dhaka, Bangladesh	Banerjee and Chandra (1992)
(Gangetic catfishes)	Buckleynema sp.	Quimperiidae	Intestine	Dhaka &/or Sylhet, Bangladesh	Bashirullah (1973) and Ahmed and Ezaz (1997)
	Cornudiscoides sp.	Dactylogyridea	_	India or Bangladesh	Chandra (2004)
	Cucullanus sp.	Cucullanidae	Stomach, intestine, body cavity	Dhaka &/or Sylhet, Bangladesh	Ahmed (1981), Bashirullah (1973) and Ahmed and Ezaz (1997)
	I. hypselobagri (uncertain species classification)	Isoparorchiidae	Swim bladder	Chittagong, Dhaka, Sylhet, Bangladesh	Bashirullah (1972),Chowdhury et al. (1986) and Chandra and Banerjee (1993)
	Procamallanus (Spirocamallanus) sp.	Camallanidae	Stomach, intestine	Chittagong, Dhaka, Sylhet, Bangladesh	Bashirullah (1973) and Ahmed (1981)
	Trypanosoma joshii (joshii not valid species)	Trypanosomatidae	Blood	Uttar Pradesh, India	Gupta et al. (2015)

Table 5Parasites identified in this study with zoonotic potential or recognised as zoonotic.

Genus, Sub Genus or Species	Host	Site of host infection	Locality human infection occurred	Zoonotic	Reference
E. heterostomum	Spotted snakehead (C. punctata)	Liver	Moirang, India	Zoonotic potential	Athokpam and Tandon (2015)
E. heterostomum	Import risk assessment <i>Oreochromis</i> spp. fillets	N/D	Imported from China or Brazil	Considered as potential zoonotic hazard in importation risk assessment	Biosecurity (2008)
E. heterostomum	North African Catfish (Clarias gariepinus)	Muscle	Buffeldoorn Dam & Seshego Dam Lebowa, South Africa	Closely related to genus, Clinostomum with zoonotic potential	Mashego and Saayman (1989)
E. heterostomum	Spotted snakehead (C. punctata)	Liver, kidney, peritoneum, muscle, and ovary	Local fish market, Aligarh, North India	High zoonotic potential Closely related species, <i>C. complanatum</i>	Shareef and Abidi (2015)
E. heterostomum	Imported Oreochromis niloticus	N/D	Imported from Eastern and Southern corridor	Included on importation risk assessment as a potential zoonosis	Walakira (2016)
Dioctophyma renale or Eustrongylides sp.	N/D	Subcutaneous nodule, chest	California, USA	Male: subcutaneous nodule chest surgically resolved. Author notes that <i>D. renale</i> has not been diagnosed in North America previously.	Beaver and Theis (1979)
Eustrongylides sp.	Largemouth black bass (Micropterus salmoides), Tench (Tinca tinca) & Sand smelt (Atherina boyeri)	Abdominal cavity and musculature	Trasimeno lake, Italy	Considered as zoonotic	Branciari et al (2016)
Eustrongylides sp.	Experimental infection	N/D	N/D	Rat: parasite survived long enough to perform extensive migrations, killing the host by damaging internal organs	Brand and Cullinan (1943)
Eustrongylides sp.	European perch (Perca fluviatilis)	Muscle	Trasimeno lake, Italy	Considered as zoonotic	Dezfuli et al. (2015)
Eustrongylides sp. (4th stage)	Minnow	Peritoneal cavity	New Jersey, USA	17-year-old male: acute abdominal syndrome surgically resolved	Eberhard et al (1989)
Eustrongylides sp. (4th stage)	N/D	Lower limbs	Southern Sudan	Women 23 and 24 years of age: cutaneous larval migration. Emergence from lower limbs	Eberhard and Ruiz-Tiben (2014)
Eustrongylides sp. (4th stage)	Minnow	Penetrating caecum and abdominal cavity	Maryland, USA	23-year-old male: Acute abdominal syndrome surgically resolved. Transverse colon ecchymotic with punctate haemorrhage and exudates	Guerin et al. (1982)
Eustrongylides sp. (4th stage)	Minnow	Cecum	Maryland, USA	25-year-old male: Acute abdominal syndrome surgically resolved. Perforated cecum	Guerin et al. (1982)
Eustrongylides sp. (4th stage)	Minnow	Unknown	Maryland, USA	Male, acute abnormal syndrome resolved without surgery	Guerin et al. (1982)
Eustrongylides sp. Eustrongylides sp.	N/D Experimental infection	Perforated intestinal wall Lumen of intestine, stomach, and penetrated wall of the digestive tract and invaded other visceral organs.	Baltimore, USA	Male: surgically resolved Rabbit: Intestinal perforation, peritonitis and multiple liver granulomas. Evidence of larval migration	Gunby (1982) Shirazian et al. (1984)
Eustrongylides sp. (4th stage)	Sushi	Peritoneal cavity	New York, USA	24-year-old male: Acute abdominal syndrome. Surgically resolved	Wittner et al. (1989)
I. hypselobagri (uncertain species classification)	Great (<i>C. marulius</i>), Striped (<i>C. striata</i>) & Spotted snakehead (<i>C. punctata</i>)	Swim bladder	Dhaka, Bangladesh	Bashirullah, 1972, stated this species can mature in fish-eating mammals, including man	Bashirullah (1972)
I. trisimilitubis adult	Crocodile	Stomach	Assam, India	Warns of human infection and any other animal that eats raw or imperfectly cooked fish. Dogs/ cats susceptible	Bhalerao (1932)

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Table 5 (continued)

Genus, Sub Genus or Species	Host	Site of host infection	Locality human infection occurred	Zoonotic	Reference
I. hypselobagri	Unknown	Unknown	Unknown	Adults expelled from humans following treatment	Chai (2019)
I. trisimilitubis adult	Wallago (Wallago attu)	Unknown	Manipur, India	Human infection reported as wide spread in Manipur villages. Reproducing adults frequently recovered from hospital patients after anthelmintic treatment. Known in villages as a human pathogen which causes choleric diarrhoea and acute abdominal syndrome.	Chandler (1926)
I. hypselobagri (uncertain species classification) juvenile	Spotted snakehead (Channa punctata)	Liver, visceral organs, fin & scales	Jaipur, India	Potential human health hazard	Mahajan et al. (1979)
I. hypselobagri (mature with eggs) however authors believed this species to be a synonym of I. trisimilitubis	Dead pig	Bile duct	Slaughter house, Mathura, India	Identified in a pig and all were active adults. Author assumed the infection was from recent consumption of infected fish.	Varma and Ahluwalia (1980)

The "Zoonotic" column includes confirmed cases or author conclusions as to the zoonotic potential of respective parasites. N/D represents not discussed in the respective publication.

3.2.2. Parasites identified in edible non-consumer ready fish, Sp. B

Phylum Platyhelminthes.

Class Trematoda.

Family Isoparorchiidae Travassos, 1922.

Isoparorchis sp. Southwell, 1913.

Two specimens were identified as *Isoparorchis* sp. based on Vankara et al. (2011) and Sohn and Na (2018). In the present study, the body is 0.30-0.40 mm long and 0.5-1.00 mm wide. The body is leaf shaped and fleshy although the tegument shows signs of damage. The oesophagus is inverted T shaped, short and the caeca bifurcates immediately at the oesophagus. The distinctive intestinal caeca undulate laterally along each side of the body and the blind ends terminate at the posterior extremity of body. The oral sucker is $0.37-0.40 \times 0.37-0.42$ mm and the VS $0.52-0.55 \times 0.50-0.55$ mm. The ventral sucker occupies the anterior half of the body and has a prominent and muscular acetabulum. The excretory pore is obvious and situated postero-terminal.

3.3. Molecular identification

Eight specimens (sample numbers 378–5; 380–1; 447–1; 477–1; 477–2; 478–1; 479–1 and 481–1) belonging to *Eustrongylides* had the 18S ribosomal RNA gene sequence of 774 bp long and showed 100% similarity with GenBank sequences assigned for *E. ignotus* (accession number AB558484) identified from Japanese smelts (*Hypomesus transpacificus nipponensis*) in Lake Biwa, Japan (Abe, 2011).

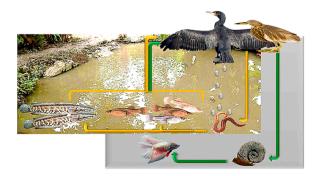


Fig. 4. The lifecycles of the nematode *Eustrongylides* spp. (yellow arrows) and the digenean *Euclinostomum* spp. (green arrows). The life cycle hosts to do not vary greatly between these two species although the parasite developmental strategies between nematodes and digeneans do. The first intermediate host for *Eustrongylides* is a range of oligochaetes and for *Euclinostomum* certain species of freshwater snails. Both species utilise small fish as 2nd intermediate hosts including a number of aquarium species for *Euclinostomum*. Predator fish have potential to become highly infected with both species of parasite. The life cycle is completed in water birds as the definitive hosts (Original figure created from images available via open creative commons). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Due to the poor quality of the chromatogram, no sequences were obtained for specimen 481–2. No sequences were available in GenBank for adult specimens of *E. ignotus* and sequences generated in the present study could not be compared. Therefore, identification of *Eustrongylides* larval type to a species level was not possible.

4. Discussion

Of the zoonotic parasites identified in this study, the nematode species *Eustrongylides* sp. was confirmed using molecular method and digeneans *Isoparorchis* sp. and *Euclinostomum* sp. using morphological method. All nematodes in the present study were larval stage and digeneans metacercarial stage which for zoonotic parasite species is the developmental stage demonstrated as infective to humans. Table 5 shows published cases of human infection associated with each parasite previously described in published literature.

Phylum Nematoda.

Class Enoplea.

Family Dioctophymidae Railliet, 1915.

Eustrongylides sp. Jägerskiöld, 1909.

At present, there are three accepted species within genus *Eustrongylides* (*E. tubifex*, *E. ignotus* and *E. excisus*) and many others of taxonomic uncertainty (Measures, 1988). *Eustrongylides* demonstrate a complex indirect life cycle (Fig. 4) that involves two intermediate hosts and piscivorous birds as definitive hosts (Agnetti et al., 2019). Morphological identification of infectious fourth stage larvae (L4) is problematic. Moravec and Nagasawa (2018) concluded that L4 stage *Eustrongylides* are unidentifiable to species by morphological features. In Mazzone et al. (2019), L4 *E. ignotus* and *E. excisus* and in Abro et al. (2020) *E. tubifex* and *E. excisus* could not be separated morphologically. It is possible therefore that species of *Eustrongylides* responsible for human infection have not yet been adequately described.

Eustrongylides, infectious L4 larvae have been reported to accidentally infect humans. The Centre for Disease Control (CDC) reported three cases, Maryland USA, of acute abdominal syndrome after minnows were consumed (Guerin et al., 1982). Two cases were surgically resolved and internal haemorrhage due to larval migration from cecum to the abdominal cavity was observed (Guerin et al., 1982; Gunby, 1982). Eberhard et al. (1989) reported acute abdominal syndrome in a human after consumption of raw fish (New Jersey, USA). Another case of human infection was reported in New York after consumption of sushi (Wittner et al., 1989). Eberhard and Ruiz-Tiben (2014) reported two cases of lower limb cutaneous Eustrongylides in South Sudan recovered during Guinea-worm testing. There are number of other non-Guinea worm parasites recovered via sampling (Eberhard and Ruiz-Tiben, 2014). It is therefore possible that cutaneous Eustrongylides may be of greater zoonotic importance than previously recognised. It is also feasible that previous reports of cutaneous Dioctophyma renale in countries where this parasite has not been widely reported, may have been misidentified. Human cutaneous D. renale has been reported in Central California (Beaver and Theis, 1979) and in Ohio (Gutierrez et al., 1989). Beaver and Theis (1979), Central Californian case, concluded, the parasite recovered could have been Eustrongylides. Certainly, L4 larvae of either species are morphologically similar and distinguished only by the position of the vulva opening (Anderson, 2000; Panesar and Beaver, 1979). In human cases of infection described in Eberhard and Ruiz-Tiben (2014), Guerin et al. (1982) and Gunby (1982) the propensity of Eustrongylides larvae to migrate was evident in the observed internal organ damage. Larval migration has also been well documented during experimental infection of rabbits (Barros et al., 2004; Shirazian et al., 1984). All Eustrongylides identified in the present study were encysted larval stage. The proven resilience of larval nematodes to certain types of processing and storage (Sánchez-Alonso et al., 2018; Wharton and Aalders, 2002) may make infectious stage Eustrongylides an unacceptable human health risk in fish imported into Australia fresh or chilled. As can be seen in Table 3, identification of Eustrongylides sp. infecting Channidae fish from the Indian sub-continent represents a new host and region record. It is therefore doubtful that this nematode species was included in Australian risk assessments for importation of Channidae fish fresh/chilled from Country 22.

Euclinostomum sp. (Rudolphi, 1809).

At present, there are five valid species within the genus *Euclinostomum* (*E. ardeolae*, *E. clarias*, *E. dollfusi*, *E. multicaecum* and *E. heterostomum*) (WORMS, 2020a). Members of this genus are a common parasite of piscivorous birds which serve as definitive hosts (Fig. 4). Metacercariae of *E. heterostomum* show little host specificity (Kazacos and Appel, 1983) and have been identified in a broad range of edible freshwater fish. Snakehead (*Channa* sp.) (Arthur and Ahmed, 2002; Vankara et al., 2011) from India and Bangladesh; tilapia (*Oreochromis* sp.) from Israel, Southern Africa and Egypt (Britz et al., 1985; Caffara et al., 2016; Taher, 2009) and tilapias from Egypt, Israel, Ghana and Nigeria (Britz et al., 1985; Caffara et al., 2016; Echi et al., 2012; Fischthal and Kuntz, 1963; Ukoli, 1966) are all fish species susceptible to infection. Multiple species of aquarium type fish have also been identified infected (Kazacos and Appel, 1983; Purivirojkul and Sumontha, 2013; Suanyuk et al., 2013).

Euclinostomum species are taxonomically close to other zoonotic Clinostomid digeneans (Park et al., 2009) however the zoonotic potential of Euclinostomum species has not been firmly established despite multiple authors referring to this genus as having zoonotic potential (Mashego and Saayman, 1989; Shareef and Abidi, 2015). Euclinostomum heterostomum has been included as a zoonotic hazard in the importation risk assessment of two exporting countries for edible fish (Biosecurity, 2008; Walakira, 2016). In support of Euclinostomum species as a zoonosis, is evidence of the shared expression of cysteine proteases which are the most abundantly expressed family of protease in zoonotic digenetic trematodes. In the closely related Clinostomum complanatum the release of cysteine proteases is not only involved in the parasites metabolic and survival function but also in the zoonotic process (Rizvi et al., 2010). Proteolytic bands of 33, 39, 45 and 47 kDa have been reported from C. complanatum (Rizvi et al., 2010). In comparison 36, 39, 43 and 47 kDa reported for E. heterostomum (Shareef and Abidi, 2014), show approximal molecular weights. Shareef and Abidi (2014) concludes that such contiguous alignment is a reflection of similar life cycles, and a shared "functional and evolutionary significance". Release of cysteine proteases has also been demonstrated in other zoonotic trematodes, Schistosoma japonicum (Dvořák et al., 2008),

Fasciola hepatica (Norbury et al., 2011) and *C. sinensis* (Kang et al., 2010). Further investigation into the zoonotic potential of *Euclinostomum* species therefore seems warranted. According to Echi et al. (2012) metacercariae can remain viable for long periods in the host and it may be that infectious metacercariae in fresh fish imported to Australia poses an unacceptable human risk.

Isoparorchis sp. Southwell, 1913.

Isoparorchis species are digenean parasites of Family Isoparorchiidae. At present, there are five valid species within Genus Isoparorchis (I. eurytremus, I. hypselobagri, I. maharashtrensis, I. tandani and I. trisimilitubis) (WORMS, 2020b) and one species I. pakistani of taxonomic uncertainty (Shimazu et al., 2014). Shimazu et al. (2014), using combined morphological and molecular method, redescribed I. hypselobagri in the native Australian freshwater catfish (Cribb, 1988) as I. tandani. Isoparorchis hypselobagri in fish of the Indian subcontinent was redescribed as I. trisimilitubis. Isoparorchis eurytremum is described in Japan/Russia and I. pakistani (taxon inquirendum) restricted to Pakistan. Shimazu et al. (2014) further commented, Isoparorchis sp. 3 occurs in a freshwater catfish Wallago attu from Bangladesh.

The life cycle information for this genus has so far been poorly described (Nagasawa et al., 2013). The most reliable description was provided by Cribb (1988) on *I. tandani* in the Australian freshwater catfish *Tandanus tandanus*. It is unknown if piscivorous birds may also be part of the parasite life cycle. Intermediate hosts include various freshwater snails (Nagasawa et al., 2013; Shimazu et al., 2014) and in Australia, atyid shrimps (Cribb, 1988). *Isoparorchis* sp. infect a broad species range. Fish susceptible to infection include *Mystus seenghala* (syn. *Sperata seenghala*), Spotted snakehead (*C. punctata*), Striped snakehead (*C. striata*), Wallago catfish (*W. attu*) in the Indian subcontinent (Choudhary et al., 2019; Shimazu et al., 2014; Vankara et al., 2011); and in Korea *Pungtungia herzi*, *Acheilognathus koreensis*, *Squalidus japonicus coreanus* and *Odontobutis platycephala* (Sohn and Na, 2018). In Japan the Japanese eel (*Anguilla japonica*), Amur catfish (*Silurus asotus*), Yellowfin goby (*Acanthogobius flavimanus*) and a cyprinid fish (*P. herzi*) have been demonstrated susceptible to infection (Nagasawa et al., 2013). Adult and metacercariae are often found in the air bladder of suitable fish hosts however metacercariae have been identified in the fins, liver, ovaries, abdominal cavity (Cribb, 1988; Mahajan et al., 1979), muscle, and skin (Sohn and Na, 2018).

Published cases of human infections with I. hypselobagri have been reported in India and China (Chai, 2019; Chandler, 1926). Humans and catfish (W. attu) infected with I. trisimilitubis, Manipur Valley, north-eastern India, have been reported as very common (Chandler, 1926). In the same study, the author noted that in this region no differentiation was made between human infection with Fasciolopsis buski and I. trisimilitubis. As F. buski is not generally found in fish it may be that the zoonotic potential of I. trisimilitubis has been underrated. Human infection with F. buski is associated with abdominal syndrome and diarrhoea. Local people in Manipur consider Isoparorchis as distinctly pathogenic, producing similar symptoms and described as choleraic diarrhoea (Chandler, 1926). Cases have also been reported in India (Faust, 1930; Mahajan et al., 1979) and from patients in Hunan Province, China (Cribb, 1988). Reproducing adult Isoparorchis have been frequently recovered after treatment from hospitalised human cases in Manipur (Chandler, 1926) and expelled adult worms after treatment were also reported by Ashford and Crewe in 2003 as cited by (Chai, 2019). A reproducing adult I. hypselobagri was identified in the bile duct of a pig (Varma and Ahluwalia, 1980). In the human cases and pig infection the authors concluded infection to be accidental consumption of reproducing adult digeneans in infected fish (Chandler, 1926; Varma and Ahluwalia, 1980). Given the frequency of viable reproducing adults expelled from hospitalised humans after treatment it does not seem logical that all patients had recently consumed infected fish. It must be considered that adult worms may survive for prolonged periods in humans and/or may develop to adults in humans. The viable, reproducing adult digenean recovered from the gall bladder of the pig infers that migration had occurred out of the stomach without demonstrable damage to the parasite. Bhalerao (1932) commented that human infection in India may occur from eating imperfectly cooked Wallago catfish (W. attu) and that dogs and cats were susceptible to infection. Bashirullah (1972) believes this species can mature in fish-eating mammals, including man in Bangladesh from consumption of striped (C. striata), great (C. marulius) and spotted snakeheads (C. punctata). As the morphology of Isoparorchis spp. is quite distinctive it seems unlikely that despite publications being dated the parasitologists at the time misidentified the genus. This parasite like Schistosoma mansoni excretes ammonia in the host (Adak and Manna, 2008). Therefore, the zoonotic potential of Isoparorchis spp. may be linked to this chemical which is highly corrosive and has potential to damage human body cells (NYS. Dep. Health, 2011). Ammonia is also converted to urea in the parasite and the high urea levels are associated with heavy infection (Adak and Manna, 2013). The toxic and zoonotic potential of Isoparorchis sp. in fresh imported fish requires further investigation particularly as parasite toxins may still cause human illness even if the parasite were dead. Although the two specimens in the present study were not identified to a species level the morphology is close to descriptions for *I. trisimilitubis* in Sohn and Na (2018). The origin of Sp. A. also increases the likelihood of all specimens being I. trisimilitubis larval stage.

4.1. Importation into Australia

Edible fish of Sp. A from Country 22 is permitted entry into Australia, at the time fish were examined, fresh/chilled, under the importation commodity code 0302. Providing fish is accompanied by a declaration from an official testing facility in Country 22 which certifies fish are compliant with Australian import conditions, according to BICON, fish are not inspected on entry into Australia or as non-risk fish inspected at a reduced rate as per the 'Imported Food Control Regulations' (Aust. Gov., 2019; BICON, 2020). Fish included under consumer ready commodity code 0302 must have the liver, intestines, head and gills removed and internal/external surfaces thoroughly washed (Aust. Gov., 2017; BICON, 2020). Williams et al. (2021) showed a significant association between presence of entire or partial intestinal contents with the occurrence of parasites in consumer ready edible imported fish. The imperfect processing standards of imported Sp. A fish is clearly a risk for entry of zoonotic/non-zoonotic parasites species into Australia. Edible imported fish of Sp. B may be imported into Australia fresh and entire (uneviscerated) providing the fish is wild caught, not farmed. Sp. B fish are listed as a commercial, aquaculture species for Country 22 in Froese and Pauly (2018) and the importation status requires further

consideration. The intestinal contents of imported Sp. B fish in present study was full of *Indoplanorbis* like snail shells. This snail is an intermediate host for *Isoparorchis* sp. (Shimazu et al., 2014). It seems clear that farm level management of gastropods may be imperfectly performed in the exporting country and this impacts the safety of the imported product.

It should be noted that all parasites in the present study were obtained from frozen fish imported into Australia and as a result the human health risk should not be overstated. Therefore, providing fish were frozen and maintained at a temperature suitable to inactivate both metacercariae and larval nematodes (Fan, 1998; Sánchez-Alonso et al., 2018; Wharton and Aalders, 2002) the human health risk has been effectively negated. However, at the time fish were examined (2020), both fish groups were permitted entry into Australia fresh or chilled which increases the human health risk from zoonotic parasites. Sp. A were consumer ready, small and likely to be prepared and consumed without further processing. Sp. B were very small and are traditionally consumed entire.

5. Conclusion

Australia places significant trust in the ability of the exporting country to provide fish which complies with international and national food safety standards. Fish imported into Australia fresh and contaminated with parasites may be an area which could be investigated further. The parasites identified in edible fish are considered to be a result of both inexperienced processing and poor farm level chemotherapy control. All families of zoonotic parasites recovered in this study have a demonstrated resilience to many types of storage and processing. It is considered, the commodity code which allows fresh fish into Australia from the exporting country (Country 22) be re-evaluated.

Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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