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Case Report

A case of cutaneous paragonimiasis presented with minimal pleuritis

T Shantikumar Singh, KH Ranjana Devi[1], S Rajen Singh[2], Hiromu Sugiyama[3]

Departments of Microbiology, Sikkim Manipal Institute of Medical Sciences, Gangtok, [1]Microbiology, and [2]Surgery, Regional Institute of Medical Sciences, Imphal, Manipur, India, [3]Parasitology, National Institute of Infectious Diseases (NIID), Tokyo, Japan

KEY WORDS

Cutaneous paragonimiasis, mild pleuritis, subcutaneous nodule

ABSTRACT

Clinically, paragonimiasis is broadly classified into pulmonary, pleuropulmonary, and extrapulmonary forms. The common extrapulmonary forms are cerebral and cutaneous paragonimiasis. The cutaneous paragonimiasis is usually presented as a slowly migrating and painless subcutaneous nodule. The correct diagnosis is often difficult or delayed or remained undiagnosed until the nodule becomes enlarged and painful and the cause is investigated. We report here a case of cutaneous paragonimiasis in a male child who presented with mild respiratory symptoms. The diagnosis of paragonimiasis was based on a history of consumption of crabs, positive specific serological test, and blood eosinophilia. The swelling and respiratory symptoms subsided after a prescribed course of praziquantel therapy.

INTRODUCTION

Paragonimiasis is one of the important food-borne parasitic zoonosis caused by trematode species of the genus *Paragonimus* which is widely distributed in the world. *Paragonimus westermani* has been the commonest species causing infection in humans in Asia; however, recently *Paragonimus heterotremus* has been increasingly detected as an important human pathogen occurring in South and Southeast Asian countries, including India.[1-3] The infection is acquired by eating raw or inadequately cooked fresh water crabs and or crayfish, which served as second intermediate host of lung fluke. Although the lungs are the primary sites of infection, *Paragonimus* can infect any organ or tissue of the body except bone. Extrapulmonary infection is due to the abnormal migration of the immature worms. Certain species such as *P. heterotremus, Paragonimus skrjabini* and *P. westermani* are well known to cause extrapulmonary infections in brain, skin, peritoneal cavity, and eye.[4] Cutaneous paragonimiasis can occur concurrently with pleural effusion or pulmonary infection. In some of the cases, cutaneous paragonimiasis may precede pulmonary or pleural infection. In other cases, cutaneous paragonimiasis may follow pleuropulmonary infection or it may occur without involving any other systems. The definitive diagnosis of cutaneous paragonimiasis can be made by the demonstration of *Paragonimus* ova and or adult worm in the excision biopsy or specific antibodies by serological test. The drug of choice for the treatment of paragonimiasis is Praziquantel, which is given in dosage of 25 mg/kg body weight in three doses per day for 3-5 days. Here we present a case of cutaneous paragonimiasis in a child because of its rarity and diagnostic confusion with other causes of benign subcutaneous swellings.

CASE REPORT

A 3.5-year-old male child from Senapati district in Manipur reported with intermittent low-grade fever,

Address for correspondence
Dr. T. Shantikumar Singh,
Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, Gangtok, Sikkim, India.
E-mail: shantikumar_singh@rediffmail.com

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occasional non-productive cough, and mild chest pain since 6 months. There was no history of contact with known case of pulmonary tuberculosis. Physical examination revealed no abnormal findings except a diffuse subcutaneous swelling on the left posterior chest wall which was detected incidentally. The swelling was first noticed on the left upper quadrant of the abdominal wall then migrated through the right anterior chest wall to the posterior surface of left chest. The nodule measuring 4 cm in diameter was firm, non-tender and without any sign of inflammation. Chest X-ray showed no abnormal features except mild pleural reaction and thickening in both sides. Mantoux test was negative. His CBC (complete blood count) showed hemoglobin-11.8%, TLC (Total leucocyt count)-14,000/cu mm, Neutrophils-60%, Lymphocytes-28%, Monocytes-2%, Eosinophils-10% and ESR (Erythrocyte sedimentation rate)- 50 mm at the end of 1st h (Westergren's method). Stool examination for parasite ova and cyst by formol ether sedimentation technique for 3 consecutive days was negative. Aspiration cytology showed eosinophilic granulomatous exudate, leucocytes, and Charcot-Leyden crystals. Further history of dietary habit revealed that the child was non-vegetarian and had consumed smoked and fried crabs collected by the parents from the local mountain streams. On clinical suspicion serological test for paragonimiasis was advised. Dot Immunogold Filtration Assay (DIGFA) test for paragonimiasis was positive [Figure 1]. The case was finally diagnosed as cutaneous paragonimiasis with pleuritis. A course of Praziquantel at 25 mg/kg body weight three times a day for 3 days was prescribed. The swelling as well as respiratory symptoms gradually disappeared after the therapy.

DISCUSSION

The primary site of paragonimiasis is the lungs; however, ectopic infection can occur with or without pulmonary infection. The factors which might contribute to aberrant migration are heavy infection, host immune status, and the adaptability of parasite species in the host. The most frequently encountered extrapulmonary forms are plural effusion followed by cerebral paragonimiasis and cutaneous infection. Clinically, cutaneous paragonimiasis is usually manifested as a non-tender migratory subcutaneous nodule, rarely the nodule may be fixed and slightly painful. Generally, the swelling appeared initially on the anterior chest or abdominal wall and then it may further migrate to the back of the chest or lower abdominal wall, pelvic region and lower limbs. Cutaneous paragonimiasis with migratory subcutaneous nodules was reported as high as 30-60% in China.[5,6] In India, Singh *et al.*,[7] have reported cutaneous infection in 7 out of 45 cases of paragonimiasis in children. Adult *P. heterotremus* worm was demonstrated in the excision biopsy of a subcutaneous nodule in a 10-year-old boy from Manipur.[8] All the cases of cutaneous paragonimiasis reported from Korea were associated with pleuro-pulmonary infection.[9] Subcutaneous nodule is usually single; however, multifocal nodules may occur.[10] The differential diagnosis of cutaneous paragonimiasis should include gnathostomiasis, sparganosis, and onchocerciasis, which also cause migratory subcutaneous swelling.[11] In the later cases, the migratory cutaneous nodules are usually associated with localized pain, pruritus, and erythema. In the present case, no medical attention was sought for the subcutaneous swelling as it was not causing any appreciable discomfort but to the mild respiratory infection. The respiratory symptoms might be due to the pleural reaction to the migrating worm (s) that had just entered the pleural cavity. The diagnosis of cutaneous paragonimiasis is often difficult, especially when the parasite and or ova are absent in the excision biopsy, and a specific serological test is not available. In these cases, diagnosis may be established by positive history of consumption of fresh water crabs in an endemic area, migratory nature of subcutaneous nodule and presence of numerous eosinophil and Charcot-Leyden crystals in the excised nodule and blood eosinophilia. In the present case, the diagnosis was confirmed by a positive DIGFA test. The DIGFA is a rapid, simple test as sensitive (98.8%) and specific (92%) as ELISA for detection of *Paragonimus* specific antibodies.[12] The kit was based on the principle of a membrane-based flow-through immunoassay technique and thus found simple and rapid. It does not require any special device and/or experienced technicians, and the results are obtained within 10 min. The test uses antigen prepared from adult *P. westermani*; it was found to be applicable in Japan to detect antibodies in the patient's serum infected with *Paragonimus miyazakii*.[13] However, if a specific serological test is available, excision biopsy may not be required for the diagnosis of cutaneous paragonimiasis. Moreover, the worm may not be present in the excision biopsy as it might have migrated to another site by the time biopsy was taken. *Paragonimus* ova are usually not present in the biopsy or aspirate of the nodule if the worm is immature or single. In the present case, the

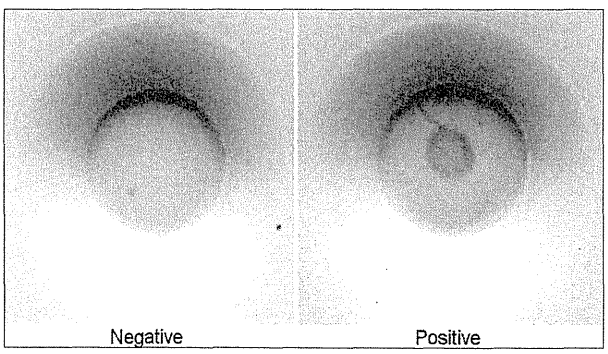

Figure 1: Dot immunogold filtration assay positive test (red circle)

Paragonimus species could not be identified as worm and ova could not be detected. However, *P. heterotremus* would be the causative agent as it is already known to exist as causative agent of human paragonimiasis in Manipur.[2]

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Review Article

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Paragonimus & paragonimiasis in India

T. Shantikumar Singh, Hiromu Sugiyama* & Achariya Rangsiruji**

*Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, Gangtok, Sikkim, India, *Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan & **Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand*

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Ever since the discovery of the first indigenous case in 1981, paragonimiasis has gained recognition as a significant food borne parasitic zoonosis in India. The data available on the occurrence of paragonimiasis, until today, may be just the tip of an iceberg as the study areas covered were restricted to Northeast Indian States. Nevertheless, the results of research on paragonimiasis in India have revealed valuable information in epidemiology, life cycle, pathobiology and speciation of Indian *Paragonimus*. *Potamiscus manipurensis*, *Alcomon superciliosum* and *Maydelliathelphusa lugubris* were identified as the crab hosts of *Paragonimus*. *Paragonimus miyazakii manipurinus* n. sub sp., *P. hueit'ungensis*, *P. skrjabini*, *P. heterotremus*, *P. compactus*, and *P. westermani* have been described from India. *P. heterotremus* was found as the causative agent of human paragonimiasis. Ingestion of undercooked crabs and raw crab extract was the major mode of infection. Pulmonary paragonimiasis was the commonest clinical manifestation while pleural effusion and subcutaneous nodules were the common extra-pulmonary forms. Clinico-radiological features of pulmonary paragonimiasis simulated pulmonary tuberculosis. Intradermal test, ELISA and Dot-immunogold filtration assay (DIGFA) were used for diagnosis and epidemiological survey of paragonimiasis. Phylogenitically, Indian *Paragonimus* species, although nested within the respective clade were distantly related to others within the clade.

Key words India - lung fluke - paragonimiasis - *Paragonimus heterotremus* - tuberculosis

Introduction

Paragonimiasis, also known as endemic haemoptysis, oriental lung fluke infection, pulmonary distomiasis, parasitical haemoptysis, parasitare haemopte, Gregarinosis pulmonum, *etc*. is one of the most important food-borne parasitic zoonoses caused by one or more of the trematode species of the genus *Paragonimus*. The disease is endemic in many parts of Asia, Africa and South America[1]. There are about 50 species of which 11 are known to cause infections in humans, *P. westermani* has been regarded as the most common and widely distributed human pathogen in Asia[2]. In South and Southeast Asia, *P. heterotremus* has been increasingly detected as an important cause of infection in humans[3]. The natural definitive hosts of the parasite comprise large varieties of wild mammals of the canidae and felidae families and humans. A wide range of fresh water snails, and crabs as well as crayfish served as first and second intermediate hosts, respectively. Humans acquire infection, commonly

by ingestion of uncooked or undercooked crustaceans containing metacercariae, the larval stage of the parasite, and rarely by ingestion of infected uncooked or undercooked meat of pig and wild boar, which serve as paratenic hosts[4,5]. The parasites primarily infect lung but extra-pulmonary infections are not infrequent. Paragonimiasis is dignosed in the laboratory by microscopic demonstration of *Paragonimus* ova in the sputum and other clinical specimens such as faeces and pleural fluid or by specific *Paragonimus* serological tests.

In the past, human paragonimiasis was not considered a problem of public health importance in India until recently. Pulmonary paragonimiasis presenting with bloody sputum or recurrent haemoptysis was generally mistaken for sputum smear negative pulmonary tuberculosis or some other serious conditions with similar symptoms. Research on *Paragonimus* and paragonimiasis has revealed that paragonimiasis is endemic in many parts of Northeast States of India and provided valuable information on the epidemiology, *Paragonimus* species, clinico-pathology, diagnosis and treatment. This review is intended to disseminate the research findings and knowledge about *Paragonimus* and paragonimiasis in India from its confinement in the research laboratories to the clinicians who are directly dealing with the patients and more importantly to raise awareness amid health educators and general population.

Historical review of *Paragonimus* research in India

Historically, at least two *Paragonimus* species were first described from India more than a century ago; namely, *P. compactus* from an Indian mongoose (*Herpetes edwardsi*) in 1859[6] and *P. westermani* from two Bengal tigers in 1878[7]. These mammals were captured in India and died in the zoological gardens in Hamburg in Germany and Amsterdam in Holland, respectively. In 1923, Vevers have described the occurrence of *P. compactus* and *P. westermani* in India[8]. In 1926, Gulati[9] had described a new lung fluke species which he named as *Paragonimus edwarsi* from a palm civet cat, *Paradoxurus grayi* in Kumaon hills, India, but it was regarded by many as the synonym of *P. westermani*[10]. Subsequently, *P. westermani* infection was described in the lungs of two domestic dogs from Malabar and Coimbatore and a panther from Coorg[11] and in a cat from Pantnagar[12]. About 40 years later Singh and Somvanshi[13] reported *P. westermani* in the lungs of two tigers (*Panthera tigris*) from the Terai area of the foothills of the Himalayas. The same species was described in the lungs of a beer cat from the zoological park in Chandigarh[14]. *P. westermani* was also isolated from the lungs of a mongoose and two tigers which had died of unknown aetiology at the National Corbett Park, India[15,16] and tigers and cats at Kanha National Park, Mandla (India) [17,18]. In spite of the fact, that paragonimiasis was common among wild mammals and wide spread in India; no record of scientific research on *Paragonimus* and paragonimiasis in India was available until late 1900s. The earlier reports were mainly focused on autopsy findings of mammalian paragonimiasis. The first epidemiological survey of paragonimiasis in India was conducted in Imphal east district, Manipur[19] during the period from 1986 and 1987. In November 1990, the first Indo-Japan joint research on *Paragonimus* and paragonimiasis was conducted in Manipur to study the life cycle, pathobiology, and morphological characterization of *Paragonimus* species, and resulted in the discovery of endemic areas of paragonimiasis, identification of crustacean second intermediate hosts and morphological characterization of *Paragonimus* species occurring in Manipur. Two fresh water crab species namely, *Potamiscus manipurensis* (Alcock, 1909) and *Maydelliathelphusa lugubris* (Wood-Mason, 1871)[20], formerly known as *Barytelpusa lugubris* which habitate in most of the mountain streams in Manipur were investigated for *Paragonimus* metacercarial infection. *P. manipurensis* was identified as the natural crab hosts harbouring at least three types of *Paragonimus* metacercariae such as *P. heterotremus*, *P. westermani* like metacercariae (large and small types) and easily excysted thin cyst walled metacercariae[21]. We have described *Paragonimus* infection in the lungs of a civet cat from Tamenglong district in Manipur[22]. The morphological features (Carmine stained) of the adult worm isolated from the worm cyst in the lungs were distinct from other species; therefore, it was named as *P. miyazakii manipurinus* n. sub spp (as a new subspecies) due to close morphological similarity with *P. miyazakii*. Subsequently *P. hueitu'ngensis*, *P. heterotremus*, and *P. skrjabini* were described from the experimental laboratory animals infected with metacercariae isolated from the crab hosts; *Potamiscus manipurensis* collected from mountain streams in Manipur[23-25]. The occurrence of *P. heterotremus* infection in crab host, *B. lugubris* (now known as *M. lugbris*) and human was reported from Arunachal Pradesh[26]. Recently, an endemic focus of paragonimiasis has been located in Nagaland due to *P. heterotremus*[27]. The crab host in Nagaland was found to be *P. manipurensis*. Tandon *et al*[28] had described the molecular characteristics and ultrastructure of *P. westermani* metacercariae isolated from *M. lugubris*

in Arunachal Pradesh. Devi *et al*[29] reported the morphological and molecular characterization of *P. westermani* adult worm isolated from the experimental Wistar rats infected with *P. westermani* metacercariae harvested from *M. lugubris* collected from Arunachal Pradesh and Meghalaya States of India and showed that Indian *P. westermani* complex represented a distinct lineage. In June 2011, we found that fresh water crab species, *Alcomon superciliosum* (Kemp, 1913) collected from the mountain streams in Moreh town in Manipur (located on the Indo-Myanmar border) were infected with at least two types of *Paragonimus* metacercariae *viz. P. heterotremus* and *P. westermani* (unpublished data).

Paragonimiasis in humans

In India, *Paragonimus* ova were detected from the sputum and faecal samples from a Chinese patient in Bombay (now Mumbai), Maharashtra in 1919, but believed the infection might have acquired from outside India[30]. Therefore, this case was not considered as an autochthonous case of paragonimiasis in India. The first indigenous case of human paragonimiasis in India was described by Singh *et al*[31] in 1981in Manipur. The *Paragonimus* ova were demonstrated in the sputum specimens and identified as ova of *P. westermani* based on morphological features only. The next case of human paragonimiasis was reported in 1984 from Maharashtra, India[32]. In 1986, Singh *et al*[33] have described 39 cases of pulmonary paragonimiasis in Manipur. Subsequently, several cases of paragonimiasis were reported from Manipur[34,35]. In an epidemiological survey of paragonimiasis conducted in Imphal east district, Singh *et al*[19] had discovered enedemic areas with an estimated prevalence rate of 6.7 per cent in Manipur. Paragonimiasis cases have been detected almost every year in Manipur but only a few have been published[36,37]. In the Northeast States of India, *P. heterotremus* has been identified as the causative agent of paragonimiasis in human[38,39] contrary to the earliest concept that *P. westermani* was the agent in Manipur.

Life cycle

The parasites utilize two intermediate hosts and complete the life cycle in wild mammals and humans as final definitive hosts.

First intermediate hosts: Fresh water molluscan species of the genera *Semisulcospira*, *Oncomelania*, *Brotia*, and *Thiara* serve as the first intermediate hosts of *Paragonimus* species in China, Japan and Thailand[2]. In India, the molluscan host has not yet been determined. *Paragonimus* ova discharged either in the faeces and/or sputum of definitive hosts reach water and hatch ciliated miracidia, which swim about to find a suitable snail host. The ova required at least two weeks to complete embryonation and hatching in water[40]. In the snail host, the miracidium develops into a mother sporocyst which produces asexually first and second generations of rediae, which finally develop into cercariae. The development from miracidium to cercariae in the snail takes about 9 to 13 wk[41,42]. Further development of the cercariae takes place in a suitable crustacean host.

Second intermediate hosts: Fresh water crab species of the genera *Potamiscus*, *Potamon*, *Paratelphusa*, *Eriocheir*, *Geothelphusa*, *Barytelphusa*, and crayfish species of the genus *Camberoides* and shrimps such as *Acrohrachium* and *Caridina* serve as common second intermediate hosts[2]. In Northeast India, *M. lugubris*, *P. manipurensis* and *A. superciliosum* were found to serve as the crab hosts of *Paragonimus*. In China a frog species, *Rana boulengeri* has been described to contain metacercariae of *P. skrjabini* and served as paratenic host[43]. The crab hosts are infected with cercariae either by ingestion of the snail hosts or free cercariae directly entering through the soft tissue of the crab host[44]. A single crab may harbour metacercariae of more than one *Paragonimus* species simultaneously. The cercariae develop to metacercariae which remained encysted in the liver, gills, intestine, skeleton muscles and sometimes, in the heart of the crab host. For further continuation of the life cycle, the metacercariae are to be taken up by a suitable definitive mammalian host.

Definitive hosts: Many mammals of Canidae and Felidae family and humans serve as definitive hosts. Tigers, civet cats, toddy cats, dogs, mongooses, *etc.* were found to serve as definitive hosts of *Paragonimus* species in India. Raccoon dogs, in Japan, served as natural reservoir hosts of *P. westermani* and *P. ohirai*[45,46]. In the Philippines and Malaysia, rats serve as natural definitive host as well as paratenic hosts[47,48]. The metacercariae ingested by the definitive hosts excyst larvae in the small intestine, penetrate through the intestinal wall and reach the abdominal cavity in 3 to 6 h[49]. The larvae migrate into the peritoneal cavity leaving behind haemorrhagic and rusty brown inflammatory exudates and then enter through the diaphragm into the thoracic cavity. Thereafter, the worms wander to find their suitable partners and usually in pairs invade the lung parenchyma to form a worm cyst where they mature to adult worms and start

laying ova. A few worms may remain in the pleural cavity or settle in organs and tissues other than the lungs. The time taken by the larval worms to become mature adults varies from one to three months or even longer in some species. Generally, the time taken from infection to laying eggs has been estimated to be from 65 to 90 days[50]. In their final hosts, the parasites may survive from 1 to 20 years despite the absence of specific therapy.

Species differentiation

Phenotypic characteristics: Traditionally, the study of the morphological characteristics of the adult worms, ova and metacercariae forms the basis of taxonomy of *Paragonimus*. The important morphological features of adult worms for species differentiation include: *(i)* shape and size of the whole mount of adult worms, *(ii)* arrangement of cuticular spines, *(iii)* relative size of transverse diameters of suckers, *(iv)* sizes, numbers and branching patterns of ovary and testes, and *(v)* shape, size and shell character of ova[2]. The shape, size, number of cysts and presence of pink granules in the body of encysted larvae are important characters for species differentiation of *Paragonimus*. Most species possess outer and inner cysts whereas some are without cyst, *e.g. P. mexicanus*. Other useful characters at the metacercarial stage include body proportions of the excysted larvae, relative size of the suckers, the anterior extent of the excretory bladder, the presence and length of oral stylet, the distribution of spines and the number and arrangement of papillae around the suckers[51-54].

Genotypic characteristics: Molecular characterization of genomic DNA of parasites can be performed using adult worms, larvae and ova. As the adult worm is not usually recovered in the clinical specimens from patients, molecular characterization of ova is the only option for accurate species identification of the causative agent. A phylogenetic analysis based on the ITS2 region of three species from Manipur and Arunachal Pradesh showed *P. westermani* from Manipur and Arunachal Pradesh in the same clade as *P. westermani* and *P. siamensis* from Thailand (Fig. 1). In addition, *P. heterotremus* from India were placed close to *P. heterotremus* from China and Thailand[38,55]. *P. skrjabini* from Manipur, was closely related to *P. skrjabini* from China and *P. miyazakii* from Japan. Blair *et al*[56,57] described that *P. skrjabini* .from eastern China (Fujian Province) was phylogenetically very close to *P. miyazaki* from Japan and proposed that both the populations should be named as the same subspecies as *P. skrjabini miyazaki*. They also considered *P. szechuanensis* as the synonym of *P. skrjabini* and concluded that *P. skrjabini* represents a separate species complex. Recently, a new lung fluke species, *P. pseudoheterotremus* morphologically similar to but genetically distinct (different nucleotide sequence in *cox1* genes) from *P. heterotremus* was described from Thailand[58,59]. This finding suggests possible occurrence of cryptic or sibling species to *P. heterotremus*.

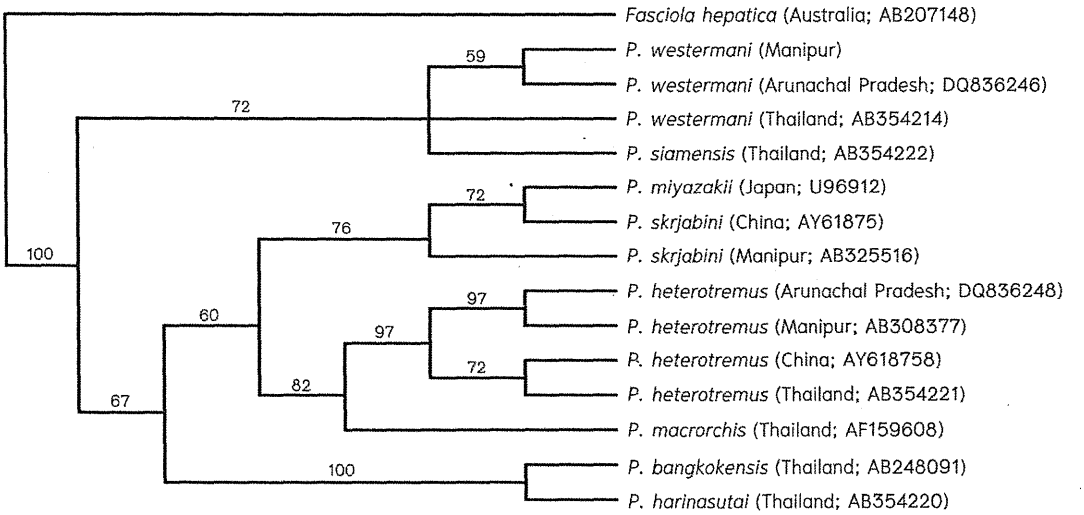

Fig. 1. Phylogenetic analysis of Indian *Paragonimus* spp. Numbers on the branches are bootstrap values (%) of 1,000 replicates. The sources of data, including geographical origins and GenBank accession numbers are indicated in parentheses.
Source: Ref. 37.

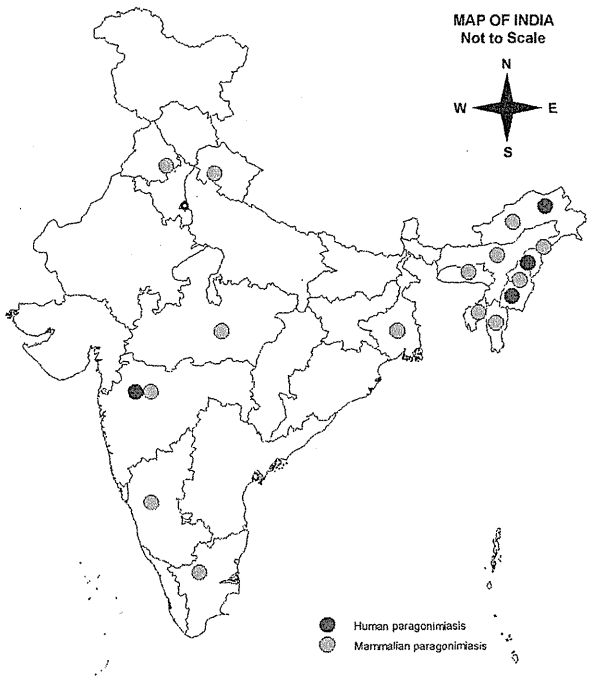

Fig. 2. Geographical distribution of paragonimiasis in India.

Experimental infection of laboratory animals

Experimental infection of laboratory animals is essential to determine the host-parasite relationship between various *Paragonimus* species and laboratory animals, to study the pathogenesis, to recover adult worms for detailed morphological and molecular characterization, to study immune responses during the course of parasite infection and to obtain antigen for developing immunodiagnostic tests. Therefore, selection of suitable laboratory animal species is a prerequisite for the successful recovery and good yield of the adult parasites. Generally, puppies and kittens are susceptible to all *Paragonimus* species in Asia and Southeast Asia and served to be the ideal animal model for experimental studies[60-63]. Puppies were found susceptible to all *Paragonimus* species occurring in Manipur[23,24,64] whereas Wistar rats were found as a suitable rodent model for paragonimiasis in Arunachal Pradesh[65].

Pathology

The pathologic lesions of paragonimiasis may be due to invasion and migration by the worms and toxic metabolites and host's immune responses to the invading parasites. Small haemorrhagic and inflammatory spots along the route of migration of the immature worm(s), in the intestine, peritoneum, abdominal organs, diaphragm, thoracic cavity, and pleura, and sometimes, pericardium had been observed in the experimental animals[23,24]. The similar observations were made by other workers and also expected to occur in humans[61,62]. The inflammation may cause adhesion between intra-abdominal organs and peritoneal wall or diaphragm. Sometimes, granuloma may be formed around ova deposited by the migrating adult worms in the abdominal cavity. The basic pathology is the formation of a worm cyst primarily in the lungs. The worm cysts varying from 1 to 3 cm in size usually located superficially, sometimes deep in the lung parenchyma contained dark red or rusty brown viscous fluid, red blood cells, inflammatory cells, eosinophils, necrotic tissue, Charcot-Leyden crystals, and adult worms and ova. The adult worms are usually found at the periphery of the cyst, adhered to the cyst wall. Generally, a cyst contained paired worms, but single or more than two worms of the same or different species may be found[54,64]. The cyst is communicated with bronchioles through which the cystic fluid, including the ova is discharged in the sputum to the exterior environment. If the sputum is swallowed as in case of young children, the ova can be detected in the faeces. Histopathologically, the worm cyst is composed of an outer fibrocollagenous cyst wall with focal haemosiderin-laden macrophages and an inner layer of congested blood vessels, inflammatory cells and normal or distorted ova.

Clinical manifestations

The incubation period may vary from 1 to 2 months or even longer. The shorter incubation period from 2 to 15 days and appearance of early symptoms were observed in 52 per cent of cases in an outbreak of *P. westermani* infection in Harbin, China[66]. Clinically, paragonimiasis may be broadly classified into pulmonary, extra-pulmonary and pleuropulmonary forms. Others have categorized paragonimiasis, as acute paragonimiasis, chronic pleuropulmonary paragonimiasis, and ectopic paragonimiasis[1].

(i) Pulmonary paragonimiasis: Pulmonary paragonimiasis is the commonest clinical form of paragonimiasis occurring in 76-90 per cent of cases[33,35]. Generally, most patients are ambulatory, apparently healthy and capable of performing normal physical activities. Initially, the patients may present with signs and symptoms of mild pleural effusion, pneumonitis, bronchiectasis or bronchopneumonia. Major presenting symptoms comprised pain or tightness in the chest,

difficult breathing, and coughing up rusty brown or blood-stained sputum or recurrent haemoptysis. Haemoptysis may be exacerbated on physical exertion, and massive requiring immediate medical attention and hospitalization. The chronic infection may be associated with fever, anaemia, weakness, and weight loss. Generally, pulmonary infection has high morbidity and low mortality unless complicated with infection in vital organs such as heart, and brain.

(ii) Extra pulmonary paragonimiasis: Extra pulmonary paragonimiasis is due to aberrant migrations of juvenile worms, which deviate from the normal route of migration to the lungs through the intestine, abdominal cavity, diaphragm and pleural cavity. It is likely to occur more commonly in children and in heavy infections. In a review of 45 cases of paragonimiasis in children in Manipur, 44.4 per cent of cases were extra pulmonary[35]. In another report, extra pulmonary paragonimiasis in adults was seen in 2 per cent of 247 cases[67].

Pleural effusion: The migrating worms in the pleural cavity produce pleuritis and effusion; the severity may vary from a minimal to massive effusion depending on the number of worms, frequency of migration and duration. Frank effusion was found in up to 20 per cent cases of pulmonary paragonimiasis in which 60 per cent bilateral, 20 per cent unilateral and 20 per cent encysted were found[32,35,19]. Uchiyama *et al*[69] reported 5 per cent bilateral and 35 per cent unilateral effusion, 45 per cent nodular or cavity lesion and 15 per cent pleuroparenchymal complex. Persistent effusion was reported in nine patients from Lao PDR[69]. The main presenting symptoms of patients with pleural effusion were progressive dyspnoea, chest pain and cough with or without haemoptysis.

Cutaneous paragonimiasis: The skin is one of the common sites for ectopic paragonimiasis which may be seen in about 16 per cent of children[35]. The typical lesion is a migratory subcutaneous nodule which usually appear on the anterior chest wall then migrating through the abdominal wall to the pelvic region or lower limbs without causing much discomfort to the patient except mild pain and itching. The abnormal migration is likely to occur in heavy infection in which the immature worm may invade any organ or tissue except bone. The factors which contribute to abnormal migration are not clearly understood, however, host specificity, and parasite species adaptability may play a major role in the pathogenesis. For example, the Chinese species *P. skrjabini* is well known to cause extra pulmonary infections in brain, skin, peritoneal cavity and eye[66]. The cutaneous infection caused by *P. skrjabini* has been estimated to be between 30 to 60 per cent of the infected patients[70]. *P. skrjabini* is also expected to be one of the causative agents of cutaneous paragonimiasis in Manipur. Seven out of 45 cases in childrenin Manipur were found to be cutaneous paragonimiasis; presented with migratory subcutaneous nodules except in one[35]. *Paragonimus* adult worm could not be isolated from the excised biopsy materials except in one case who presented with a subcutaneous swelling on the right anterior chest wall. The carmine stained worm was morphologically identified as an adult *P. heterotremus*[71]. Generally, the subcuataneous nodule is non tender, mobile, soft to firm in consistency, round to oval measuring 2-5 cm in diameter, and is usually not associated with obvious signs of inflammation or creeping eruption. Rarely, the nodule may remain localized without further migration. The immature or adult worm along with inflammatory exudates, granulomatous tissue, eosinophils, Charcot-Leyden crystals and sometimes *Paragonimus* ova may be detected in the excision biopsy of the nodule if performed before further migration of the worm.

Cardiovascular paragonimiasis: This is a rare but serious condition which may be associated with pleuropulmonary paragonimiasis. We have described three cases of pleuropulmonary paragonimiasis in children involving heart[35]. Two of them presented with pericarditis and one child who presented with pericarditis, and myocarditis died of congestive heart failure in spite of praziquantel therapy.

Abdominal paragonimiasis: The symptoms during the prepatent period are usually mild that majority of patients do not recall if they had any gastrointestinal symptoms prior to pulmonary paragonimiasis. Some patients might present with vague abdominal pain, nausea, vomiting, mild diarrhoea and allergic dermatitis. Rarely, patient may present with diarrhoea and dysentery[33] and abdominal distension with hepatomegally[35].

Cerebral paragonimiasis: Cerebral paragonimiasis has been described as the most common and serious form of extra-pulmonary paragonimiasis, which was reported mainly from China[70], Japan[72], and Formosa[73,74]. The infection has been described due to erratic migration in which the worms enter the cranial cavity through the jugular or carotid foramen and commonly invading the temporal and occipital lobes[75]. The condition may be

confused with tubercular meningitis or tuberculoma or other space occupying lesions in the brain. The cerebral paragonimiasis is commonly seen in young adults who may present with symptoms of convulsive seizures, especially Jacksonian type, and may be associated with fever, headache, nausea, vomiting, visual disturbance, and motor weakness[76]. In India, the first case of cerebral paragonimiasis mimicking tuberculoma in a child was reported from Nagaland[77]. The imaging techniques such as X-ray, CT scans, and MRI are used for specific diagnosis of cerebral paragonimiasis[78].

(iii) Pleuropulmonary: The pleuropulmonary paragonimiasis usually occurs when both pleura and lung parenchyma are affected concomitantly. Most patients in the early stage of pleural infection show symptoms of pleurisy and pleural effusion of varying severity followed by parenchymal infection. The clinical manifestations are pain and tightness in the chest, dyspnoea, coughing up blood-stained sputum or haemoptysis and may be associated with fever. The condition was seen in 2.2 per cent of paragonimiasis patients in Manipur[33]. A high seroprevalence (31.6%) of pleuropulmonary paragonimiasis was also reported from Arunachal Pradesh[39].

Laboratory diagnosis

A definitive diagnosis can be made by finding characteristic golden brown, ellipsoidal or oval operculated *Paragonimus* ova in the clinical specimens such as sputum, aspirated fluids and faeces by microscopy. Occasionally, adult worm may be detected in the excised tissue or cyst or at necropsy or pleural aspirate[69] or expectorated in the sputum[79]. When the ova or worms are not detectable, serological tests play an important but adjunctive role for the diagnosis of extra pulmonary paragonimiasis.

Microscopy

Sputum: Morning sputum samples are ideal for a direct wet smear examination for the parasite ova. The rusty brown or blood-stained sputum usually contains numerous *Paragonimus* ova and Charcot-Leyden crystals. Earlier studies showed *Paragonimus* ova in 55.6 to 72 per cent of sputum specimens of pulmonary paragonimiasis cases[33,80]. Devi *et al*[37] found ova positive sputum in 20.9 and 4.1 per cent of pleuropulmonary paragonimiasis in children and adults, respectively. The finding, however, is unusual because sensitivity of sputum examination is expected to be higher in adults than in children.

Pleural fluid: *Paragonimus* ova may be demonstrated in the centrifuged deposit of pleural fluid in about 10 per cent cases of pleural effusion[81]. Vidamaly *et al*[69] demonstrated *Paragonimus* ova in all the pleural aspirats of nine patients with persistent effusion and one of them discharged an adult worm through a draining chest tube. In the absence of demonstrable ova pleural fluid analysis showing glucose content less than 10 mg/dl, lactose dehydrogenase greater than 1000 IU/l, high-protein value, low *p*H and eosinophilia is indicative of pleuropulmonary paragonimiasis[82].

Stool: Stool examination for *Paragonimus* ova is recommended in children who usually swallow sputum and in patients whose sputum samples are apparently negative for ova. Ideally, two to three stool samples collected at consecutive days should be examined by formolin-ether sedimentation or AMS III concentration technique. The recovery rate varied from 25.6 to 60 per cent, and is higher in children <10 yr of age. Komiya and Yokogawa[80] reported *Paragonimus* ova in 65.1 per cent in the faecal samples of 189 patients of pulmonary paragonimiasis by AMS III concentration technique.

Biopsy: Excision biopsy served as surgical treatment of subcuateous nodule as well as laboratory diagnosis. Adult or immature worm may be found in a carefully dissected nodular or cystic lesion. Microscopic examination of the exudates will show inflammatory cells, eosinophils, *Paragonimus* ova and Charcot-Leyden crystals. Alternatively, histopathological section will reveal fibrocollagenous tissue, inflammatory cells, eosinophils, sections of the worms and distorted ova.

Immunodiagnosis

The serological tests are important in the diagnosis of extra pulmonary paragonimiasis and to differentiate it from tumours and cysts or nodular lesions caused by other parasites. Some of these tests are used by other researchers for evaluating the therapeutic responses to specific chemotherapy. The various immunological tests which have been evaluated are intradermal (ID) test, complement fixation test (CFT), immunodiffusion, indirect haemagglutination test (IHA), enzyme-linked immunosorbent assay (ELISA), dot-ELISA, and Western blot. ID test is simple, easy to perform, cheap, rapid and highly sensitive and was widely used over the past several years in Japan[83,84], China[85] and in India[19] for mass screening and laboratory diagnosis. It is a valuable test in distinguishing pulmonary paragonimiasis from pulmonary tuberculosis, especially in the areas

where both are co-endemic. The test is performed by intradermal inoculation of 0.01 to 0.1 ml of the test antigen (saline extract or purified antigenof adult *P. westermani*) on the volar aspect of the forearm, and the wheal diameters are measured immediately and 15 min after the inoculation. A differential wheal diameter of ⩾5 mm with erythema and pseudopodia indicates a positive test. Exceptionally, a large erythema of ⩾45x35 mm in diameters without an appreciable wheal and pseudopodia may indicate a positive reaction. Whereas a negative skin test almost certainly rules out paragonimiasis, a positive test cannot differentiate between the past and the present infection as the test may remain positive as long as 10 to 20 years even after the successful chemotherapy or spontaneous recovery[86]. There is also possible cross-reaction with other trematode infections such as schistosomiasis and clornorchiasis, if a crude antigen is used. However, the sensitivity and specificity of the test can be upto100 per cent by using purified antigen[87].

CFT has been used in the diagnosis of active infection and to confirm ID positive cases. The test becomes negative within 3 to 9 months after successful treatment[87]. It was recommended that ID test where used should be applied first in the epidemiological survey and followed by CFT or any other more specific test on individuals who showed positive or doubtful dermal reactions.

Biguet *et al*[87] first developed the immunodiffusion method for the diagnosis of paragonimiasis. Double immunodiffusion technique (Ouchterloney method), immunoelectrophoresis, and counter-current immunoelectrophoresis were reported to be highly sensitive and specific and can be used for speciation by demonstration of specific precipitin bands[88-90]. IHA is another simple, rapid and sensitive test. In Thailand, the test revealed asensitivity of 88 per cent in the diagnosis of paragonimiasis heterotrema[91].

ELISA test for paragonimiasis was first developed by Quicho *et al* in Thailand[92]. Since then ELISA based on different techniques and with different antigen preparations have been developed and evaluated for diagnosis of paragonimiasis[93-96]. The overall specificity of IgG ELISA using the saline extract of adult worms as an antigen was found to be 97 per cent. A 100 per cent sensitivity and specificity could be obtained in an indirect ELISA using F1 antigen fraction to detect antibody against *P. heterotremus* infection[97]. The various antigenic components such as 27 kDa possibly excretory/secretary (E/S) products of *P. westermani*[98] and 31.5 kDa substance of *P. heterotremus* were used as antigens for specific diagnosis of paragonimiasis and for serodiagnosis of human paragonimiasis heterotrema and therapeutic evaluation of praziquantel therapy[99], both by ELISA and Western blot, respectively. An enzyme-linked immunoelectrotransfer blot was developed for differential diagnosis between *P. heterotremus* and *P. westermani* infections[100]. Other ELISA techniques are sandwich ELISA using monoclonal antibodies-based antigen detection assay[101] and multiple dot-ELISA[95] now used in Japan. Generally, ELISA tests are used to detect parasite specific IgG antibodies but the detection of specific IgE antibodies was proven to reduce cross-reactions with other trematode infections and detection of parasite specific IgM antibodies was recommended in the diagnosis of infection at the early stage[102,103]. In India, Regional Medical Research Centre (RMRC, ICMR), Dibrugarh had developed IgG ELISA using E/S antigen for diagnosis of paragonimiasis and was reported to be highly sensitive and specific[104]. The ELISA tests are now most widely used for serological diagnosis of paragonimiasis due to their high sensitivity and specificity. The tests are also applicable to mass screening. However, ELISA tests are more expensive, time-consuming and require costly equipment and experienced persons and all the reagents, antigens, in particular, are not commercially available.

Rapid test: Recently, dot-immunogold filtration assay (DIGFA) kit was developed in China for anti-*P. westermani* antibody detection. This is found to be simple and rapid and does not require any special devices and/or experienced technicians and the results are obtained within 10 min. This kit was prepared using *P. westermani* antigen and reported in China to have the sensitivity and specificity up to 99 and 92 per cent, respectively[105].

Haematological investigation

Leucocytosis with relative lymphocytosis, eosinophilia and increased ESR were common findings in patients with paragonimiasis[33]. Most patients have haemoglobin value within normal limits in spite of frequent spiting of blood and recurrent haemoptysis. Eosinophilia (absolute eosinophil count >1000/μl) and increased ESR upto 104 mm at the end of 1st h (Westergren) were consistently found in children with paragonimiasis in Manipur[35].

X-rays and other imaging technique

The common radiographic findings were patchy air-space consolidation or opacity with associated pleural reaction or thickening, cystic or cavitary lesions, pleural

effusion (usually bilateral) and nodular opacities, which were difficult to differentiate from the similar lesions of tubercular origin. The chest radiographs showed patchy consolidations in 62-71 per cent, pleural thickening in 28 per cent, cystic or cavitary lesions in 11-14 per cent, effusion in 9-10 per cent, and nodular lesions in 8-13 per cent[33-35]. Persistent pleural effusion in nine patients was reported from Lao PDR of whom in 44.4 per cent patients showed bilateral effusion[69]. Compared to the chest X-ray, computerized tomography (CT) was found as a better technique for visualization of the lesions in the lungs[106]. Burrows and tunnels joining the cystic lesions have been described in broncho-tomogram or pulmonary CT[107,108]. Chest radiographs may be normal in symptomatic patients[31,32]. Higher rate of normal chest roentgenograms in pulmonary paragonimiasis was also reported from endemic areas in Nigeria[109]. CT and MRI of brain of cerebral paragonimiasis patients will show conglomerates of multiple ring-shaped shadows called the 'grape cluster' or 'soap bubble appearance'[110,111] or isodense lesion mimicking tuberculoma[77] in one hemisphere of the cerebral cortex.

Treatment

Three major antihelminthic drugs are currently available for treatment of paragonimiasis. Praziquantel is the drug of choice for both pulmonary and extra pulmonary paragonimiasis[112]. The recommended dose is 25 mg/kg body weight administered orally three times a day after meals for three days without any appreciable side effects. With this regimen relapse occurred in about 2 per cent cases. A 100 per cent cure rate was obtained when the therapy was extended up to 5 days[34]. Bithionol, 2, 2′-thiobis [4, 6-dichlorophenol] was the drug used before the praziquantel was available for the treatment of paragonimiasis. It is given in doses of 40 mg/kg body weight in two equally divided doses on alternate days for a course of 10 to 20 doses[33]. The most common side effect was urticaria, which was observed in about 50 per cent of patients after 2 or 3 doses of bithionol. Rarely, urticaria may be very severe requiring hospitalization and parenteral antihistaminics.The other side effects such as nausea, vomiting, diarrhoea and itching were generally few, mild and transitory. Zhong *et al*[113] had used bithionol to treat paragonimiais in doses of 50 mg/kg body weight per day on alternate days for 20 doses and found a cure rate of 97.1 per cent. Recently, triclabendazole {5-chloro-6 (2,3-dichlorophenoxy)-2methylthio benzimidazole}, a drug used in veterinary medicine was evaluated for the treatment of paragonimiasis in humans. Control trials have shown that triclabendazole when administered in a single dosage of 10 mg/kg body weight had comparable efficacy, safety and tolerability with praziquantel[114,115].

Conclusion

In the recent years, paragonimiasis has emerged as an important food-borne parasitic disease in India, mainly in the Northeastern States of India. Failure to recognize pulmonary paragonimiasis has resulted in over diagnosis of pulmonary tuberculosis and unwarranted antitubercular therapy, which will have a negative impact on the outcome of the Revised National Tuberculosis Control Programme, especially in the tuberculosis endemic areas. Mahajan emphasized the need to generate awareness among the clinicians and public regarding paragonimiasis and to consider this disease in the differential diagnosis of PTB in places where both co-exist[116]. *P. heterotremus* has been identified as the causative agent of human *paragonimiasis* in the northeast India. *Potamiscus manipurensis, M. lugubris* and *A. superciliosum* were identified as second intermediate crab hosts of *Paragonimus* in these regions. Further research work is required to determine the first intermediate snail hosts of *Paragonimus* species in India and the role of *P. skrjabini* and *P. westermani* in human paragonimiasis. The control strategies for paragonimiasis should include the epidemiological surveys to determine the magnitude of the problem, training of public health care providers about the diagnosis and management of paragonimiasis, screening of all patients attending TB clinics, DOTS microscopy centers, hospitals and rural health centers for both tuberculosis and paragonimiasis. People should be educated not to consume fresh and improperly cooked crabs and crayfish, and to clean hands, utensils, cutlery boards, strainers, knives, *etc.* after processing fresh crabs and crayfish. Public health authority should ensure the continuous supply of praziquantel in the hospitals and dispensaries. The problem of paragonimiasis is likely to continue in India unless awareness among public and medical practitioners is spread and appropriate control measures taken.

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Reprint requests: Dr T. Shantikumar Singh, Professor & Head, Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, Gangtok 737 102, Sikkim, India
e-mail: shantikumar_singh@rediffmail.com

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Short communication

Tolerance to low temperatures of *Toxocara cati* larvae in chicken muscle tissue

Kensuke Taira[a], Yasuhide Saitoh[a,*], Natsuki Okada[a], Hiromu Sugiyama[b], Christian M.O. Kapel[c]

[a] *Laboratory for Parasitology, Department of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan*
[b] *Department of Parasitology, National Institute of Infectious Disease, 1-23-1 Toyama, Shinjyuku, Tokyo 162-8640, Japan*
[c] *Section for Zoology, Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark*

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ABSTRACT

Infectivity of *Toxocara cati* larvae in muscle tissue of chickens after storage at 4 °C and −25 °C was assessed in a mouse bioassay to provide information on the risk of meat-borne toxocarosis. Muscle tissue samples of 30-day old *T. cati* infections were stored at 4 °C for 14 and 28 days and at −25 °C for 12, 24 and 48 h, whereafter, larvae were released by digestion. For each experimental group, the released larvae were inoculated in six mice. After 15 days, mice were euthanized and larval burden was assessed by digestion. In the control group (no storage of the infected chicken meat), 47.9% of the inoculated larvae established in mice, whereas storage of meat at 4 °C for 14 days or 28 days reduced the recovery to 24.1% or 3.3%, respectively. Muscle larvae exposed to −25 °C for 12, 24 or 48 h did not establish in the mice. The observation that larvae retain infective after refrigeration at exposure in 4 °C for 28 days, emphasize the zoonotic potential of poultry meat as a causative agent of human toxocarosis.

1. Introduction

The common roundworm of cats, *Toxocara cati* is a zoonotic helminth which is increasingly recognized as an etiological agent of human toxocarosis (Shimokawa et al., 1982; Fisher, 2003; Akao and Ohta, 2007; Lee et al., 2010). In addition to direct infection from eggs deposited to the environment, cultural dietary preferences for raw or undercooked chicken dishes may be associated with risk because chicken may serve as a paratenic host (Okoshi and Usui, 1968; Taira et al., 2011). Thus, accumulating evidence links human toxocarosis to consumption of raw or undercooked chicken products (Mitsugi et al., 1988; Nishikata et al., 1991; Yoshikawa et al., 2010). An experimental study clearly demonstrated that *T. cati* larvae in the chicken meat were highly infective to recipient mice (Taira et al., 2011).

As refrigeration or freezing of chicken products are common means of preservation through the consumer chain, an assessment of risk associated to consumption of *T. cati* infected chicken meat should include controlled experimental studies on the survival of muscle larvae at low temperatures. Although a few studies have indicated the survival of larvae of *Toxocara canis*, the round worm of dogs, at low temperatures, comparable knowledge does not exist for *T. cati*. Sprent (1953) reported that motile larvae of *T. canis* were found after digestion of mice carcass which had been kept at −20 °C for 4 weeks, although the infectivity of the larvae was not evaluated. Taira et al. (2004) reported also on *T. canis* that 43% of larvae from pig tissues and 19% of larvae from chicken tissues, both tissues were preserved at 4 °C for 1 week, were established in recipient pigs. In the present study, therefore, the infectivity of *T. cati*

* Corresponding author. Tel.: +81 42 850 2483; fax: +81 42 850 2483.
E-mail address: saitohya@azabu-u.ac.jp (Y. Saitoh).

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muscle larvae of chicken after storage at 4 °C and −25 °C was investigated in a mouse bioassay.

2. Materials and methods

2.1. Parasite isolates

Eggs of *T. cati* obtained from a naturally infected local cat were subsequently propagated in a laboratory cat. Adult worms expelled from this cat had typical arrow-head-formed cervical alae of *T. cati*. Fecal eggs were isolated, embryonated and stored in 1% formalin at 25 °C for approx. 3 months (Taira et al., 2011). The eggs were washed twice with tap water prior to inoculation to remove the formalin.

2.2. Chickens: infection and necropsy

Ten parasite-naive *Boris brown* chickens of both sexes, aged 4 weeks on the day of inoculation, were group-housed in disinfected cages. Food and water were administered *ad libitum*. Each chicken was inoculated orally with 10,000 embryonated *T. cati* eggs in a single dose of 0.7 ml egg suspension. All chickens were euthanized and necropsied at 30 days post-infection.

The pectoral muscles (white meat) and hindlimb muscles (red meat) of chickens were removed, and minced into pieces of approx. 5 mm^3 with a commercial food processor.

2.3. Preservation of the chicken muscle tissue

The entire portion of thoroughly mixed minced muscle tissue was divided into 18 portions of 50 g in plastic bags. All portions of minced muscle tissues in the plastic bags were flattened to about 2 cm in thickness. Three control bags were not stored and the muscle larval burden was assessed at the day of necropsy by HCl-pepsin digestion (see below). Five sets of three bags were stored respectively at 4 °C for 14 days, at 4 °C for 28 days, at −25 °C for 12 h, at −25 °C for 24 h, and at −25 °C for 48 h. The bags stored at −25 °C for 12 h were followed by 4 °C storage for another 12 h to digest the meats at the same time with that stored at −25 °C for 24 h. After storage, muscle larvae were immediately released by digestion as described below.

2.4. Mice: infection and necropsy

Thirty six 5 wk old male ICR mice (closed colony) were used in the study: Six groups of six mice were inoculated *per os* by stomach tubation with around 50 larvae per mouse (in approx. 0.5 ml saline). Inoculation was conducted within 1 h after the release of the muscle larvae. Fifteen days after inoculation, mice were euthanized. Whole carcass of a mouse, excluding the skin, tip of limbs, tail, muzzle, stomach, and intestines, were minced into pieces of approx. 5 mm^3 with a commercial food processor, and digested for larval recovery. The food processor was disinfected and washed with boiling water in the process for each carcass.

All animals used in this study were treated in accordance with the guidelines for animal experimentation of Azabu University with the reference number 100304-5 and the relevant ethical guidelines of the Japanese Ministry of Education, Culture, Sports, Science and Technology.

2.5. Digestion and larval counts

The muscle digestion was conducted after Taira et al. (2011): Chicken muscle tissue or carcass of mouse was digested in an HCl-pepsin solution for 2 h. The ratio between tissue (g) and fluid (ml) was approximately 1:10. The digestion solution was sedimented for 1 h and washed in 40 °C saline three times. Immediately after the final sedimentation, the recovered larvae were counted under a stereoscopic microscope, and their motility was roughly estimated.

3. Results

No clinical signs of the infection or changes in behavior were observed in the chickens and mice during the experiments. Macroscopic changes in muscles of chickens were not observed at necropsy.

The larval recovery from chicken meat and mice is presented in Table 1. Almost all larvae recovered from the chicken muscle tissue at necropsy (controls) were motile, and half (47.9%) of these larvae were recovered from the inoculated mice. Although, the muscle tissue did not show clear visible signs of degradation after storage at 4 °C for 14 days, only half of the released larvae were estimated as motile, and 24.1% of the inoculated larvae established in mice. After further storage at 4 °C for a total of 28 days, the tissue showed clear signs of degradation (appearance and smell), only a few of the released larvae were motile and overall only 3.3% of the inoculated larvae could be recovered from mice.

If frozen at −25 °C for 12 h, only a few of the larvae released from the chicken muscle tissue were motile, but none established in mice. When the storage period at −25 °C was extended to 24 or 48 h, the released larvae were neither motile nor infective to mice.

4. Discussion

The present study provides the first data on the infectivity of *T. cati* larvae in poultry after refrigeration or deep freezing of the meat. The bioassay in mice illustrates that *T. cati* larvae may retain high infectivity in chicken meat stored at 4 °C up to two weeks. An extended storage period significantly decreases larval infectivity. The decrease of larval infectivity may relate to decay process of the chicken meat, although the meats stored for 14 days did not show clear visible degradation. For human consumption, storage of fresh poultry would rarely exceed two weeks, and the present storage at 4 °C for 28 days may primarily be of academic interest. Thus, if stored in a common domestic refrigerator (4–5 °C), chicken meat infected with *T. cati* larvae may pose a food safety risk.

Contrary, deep freezing appears to be an effective measure to inactivate *T. cati* larvae in chicken meat, as none of the larvae recovered after freezing at −25 °C were able to establish in mice. The observation of a few motile

Table 1
Infectivity of *Toxocara cati* muscle larvae in chicken meat stored at 4 °C or −25 °C.

Chicken muscles[a]			Mice				
Storage temperature and period[b]	Number of recovered larvae in 150 g	Estimation of motility of recovered larvae[c]	N	Number of larvae inoculated per mouse	Number of larvae recovered 15 days post inoculation		Recovery % (recovered/inoculated)
				Mean (95 CI)	Mean	(SD)	Mean (%)	(SD)
0 days	1190	Approx. 100%	6	48.0 (6.07)	23.0	(4.3)	47.9	(8.9)
4 °C, 14 days	969	Approx. 50%	6	52.5 (9.86)	12.7	(2.5)	24.1	(4.8)
4 °C, 28 days	550	Approx. 5%	6	50.0 (8.26)	1.7	(1.2)	3.3	(2.4)
−25 °C, 12 h (+4 °C, 12 h)	769	Approx. 5%	6	49.0 (7.08)	0	(0)	0	(0)
−25 °C, 24 h	478	0%	6	44.8 (9.11)	0	(0)	0	(0)
−25 °C, 48 h	628	0%	6	51.8 (5.86)	0	(0)	0	(0)

[a] Pectoral and hindlimb muscles of 10 chickens inoculated with 10,000 embryonated eggs of *Toxocara cati* and necropsied 30 days after inoculation.
[b] Prior to storage, plastic bags with 50 g minced muscle tissue were flattened out to about 2 cm thickness.
[c] Motility of larvae was estimated under microscope within 1 h after release from chicken muscle tissue digested.

but non-infective larvae released from meat frozen for 12 h may illustrate that larvae may survive more moderate freezing, *e.g.* at −10 °C or −18 °C. Although the minced meat samples were flattened out, the inside temperature was not monitored in the present study, and the core temperature of the samples may not have reached a thermal dead point for the larvae within the first 12 h. Wharton and Aalders (2002) reported for fish-borne *Anisakiasis* that freezing at −20 °C for 24 h is require for the complete inactivation of *Anisakis* larvae, because a low core temperature of the fish meat is needed. Similarly, inactivation of *Trichinella* in pork by freezing is closely correlated to the size of the cut of meat and temperature and period of freezing (Noeckler and Kapel, 2007). Thus, to explore the food safety implications of the present findings, further studies are required to determine specific thermal dead point relations between size of the chicken meat, temperature and period of freezing.

In conclusion, the present study demonstrated that *T. cati* larvae in chicken muscle tissue were highly infective even after 14 days refrigeration at 4 °C, but also that deep freezing appear to be an effective inactivation measure. The results indicate that *T. cati* in poultry may pose a food safety risk if infected chicken is consumed raw or undercooked without prior deep freezing, and thus support the assumption that *T. cati* in poultry may be an agent of human toxocarosis.

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