39 Paragonimus

Hiromu Sugiyama, Takhellambam Shantikumar Singh, and Achariya Rangsiruji

CONTENTS

39.1 Introduction 423
39.1.1 Classification and Morphology of *Paragonimus* spp. 424
39.1.1.1 *Paragonimus westermani* (Kerbert, 1878; Braun, 1899) 424
39.1.1.2 *Paragonimus skrjabini* (Chen, 1959) and *Paragonimus miyazakii* (Kamo et al., 1961) 425
39.1.1.3 *Paragonimus heterotremus* (Chen and Hsia, 1964) 425
39.1.1.4 *Paragonimus africanus* (Voelker and Vogel, 1965) 425
39.1.1.5 *Paragonimus uterobilateralis* (Voelker and Vogel, 1965) 425
39.1.1.6 *Paragonimus mexicanus* (Miyazaki and Ishii, 1968) 426
39.1.1.7 *Paragonimus kellicotti* (Ward, 1908) 426
39.1.2 Biology and Pathogenesis 426
39.1.3 Clinical Features 426
39.1.3.1 Pulmonary Paragonimiasis 426
39.1.3.2 Extrapulmonary Paragonimiasis 426
39.1.3.3 Pleuroplumonary Paragonimiasis 427
39.1.3.4 Differential Diagnosis Based on Clinical Symptoms 427
39.1.4 Diagnosis 427
39.1.4.1 Conventional Techniques 427
39.1.4.2 Molecular Techniques 429
39.2 Methods 430
39.2.1 Sample Preparation 430
39.2.1.1 Eggs 430
39.2.1.2 Adult and Immature Flukes 430
39.2.1.3 Metacercariae 431
39.2.2 Detection Procedures 431
39.2.2.1 Reagents for PCR 431
39.2.2.2 PCR Amplification and Visualization of the Amplified Products 432
39.2.2.3 PCR-RFLP Analysis 432
39.2.2.4 Cycle Sequencing 432
39.3 Conclusions and Perspectives 432
References 432

39.1 INTRODUCTION

The lung flukes of the genus *Paragonimus* cause infection primarily in the lungs and pleural cavity. This disease entity is called pulmonary paragonimiasis, which is difficult to differentiate from other respiratory diseases, especially pulmonary tuberculosis, due to its similar clinical and radiological features [1]. Except in highly endemic areas, paragonimiasis is never considered a public health problem, and misdiagnosis and mismanagement of pulmonary paragonimiasis are common. In addition, extrapulmonary paragonimiasis has also been reported as an important clinical manifestation [2].

About 50 species of *Paragonimus* trematodes (Distoma), including synonyms, are distributed in tropical, temperate, and sub-Arctic zones worldwide. Human cases of paragonimiasis occur mainly in countries with traditions of eating raw or undercooked second intermediate hosts, freshwater crabs, or crayfish. Consumption of raw or improperly cooked meat of paratenic hosts, such as wild boar, is another possible source of infection [3,4]. Through these types of food practices, it is estimated that about 293 million people are at risk for infection with *Paragonimus* spp. and several million people are infected worldwide [5,6].

The identification of *Paragonimus* spp. has relied on morphological characters of adult flukes and metacercariae, and sometimes eggs [7]. Recently, extensive studies of various *Paragonimus* spp. have been carried out using molecular techniques based on PCR and nucleotide sequencing. The generated DNA sequences have allowed the identification to the species level for specimens at any developmental stage. These sequence data are also used in taxonomic studies [8], improving our understanding of epidemiology

of paragonimiasis. In this chapter, we provide an overview of the morphology and taxonomy of the important *Paragonimus* spp. that are involved in human disease. In addition to clinical manifestations in patients, diagnostics involving immunological and molecular techniques are also reviewed.

39.1.1 Classification and Morphology of *Paragonimus* spp.

Traditionally, morphological characterization has been regarded as an important criterion for identifying and establishing the taxonomy of the *Paragonimus* spp. Much emphasis was placed on the morphological features of adult worms at the macroscopic and light microscopic levels, including body shape and size, relative size of the oral sucker to the ventral sucker, patterns of lobulation of the ovary and testes, and arrangement of cuticular spines. At the metacercarial stage, morphological characteristics such as the presence of an inner and/or outer cyst wall(s), thickness of each cyst wall (if present), relative diameter of suckers, anterior extent of the excretory bladder, presence and length of a stylet on the oral sucker, presence of colored granules in the body, number of flame cells, body spination, and arrangement of papillae around suckers were employed as the key for species identification. At the egg stage, the size and eggshell sculpturing were the important keys for species identification and discrimination [6,7]. However, the extent of variation in morphology of adult worms, metacercariae, and eggs even among a single species [6] makes taxonomic classification difficult.

During the last decade, extensive studies of *Paragonimus* have been carried out using advanced molecular techniques based on PCR and DNA sequencing. Accumulated nuclear and mitochondrial sequence data have been employed to discriminate and identify the *Paragonimus* spp. Subsequent additions of sequence data for *Paragonimus* spp. have permitted reevaluation of the phylogenetic relationships of *Paragonimus* spp. [8–10]. Blair et al. used molecular phylogenetic analyses to divide *Paragonimus* spp. into a number of major groups, namely *P. westermani, P. skrjabini P. heterotremus*, *P. mexicanus* and *P. ohirai* [10]. Even though the number of valid species in the genus *Paragonimus* remains controversial, it is clear that the following seven species (or species-complexes) are causative agents in human infection [4,6].

39.1.1.1 *Paragonimus westermani* (Kerbert, 1878; Braun, 1899)

P. westermani is the type species of the genus that was first described by Kerbert in 1878 based on adult worm specimens obtained from the lungs of a Bengal tiger that died at the Amsterdam Zoo in 1877 [4,9]. The adult fluke is oval in shape. Its surface is covered with singly spaced cuticular spines. The oral sucker is usually slightly larger than the ventral sucker. The testes are divided into five or six lobes, while the ovary is simply branched into six lobes. The metacercariae are spherical, 280–450 μm in diameter depending on region of origin [4]. It has thick inner and thin outer cyst walls. Larval body is expanded to the entire cyst.

P. westermani is widely distributed from East Asia (China, Taiwan, Korea, Japan, and Southeast Siberia), Southeast Asia (the Philippines, Malaysia, Thailand, and Vietnam) to South Asia (Sri Lanka and India). Different populations isolated from various geographical regions show differences in animal and/or human susceptibilities [4,6]. Confirmed cases of human infection have been reported only from East Asian countries and the Philippines. Among *P. westermani* isolates, different ploidy levels, including diploid, triploid, and tetraploid, have been observed [9]. The triploid form has been reported only from East Asian countries and is regarded as being more pathogenic in humans [12]. The eggs of triploids are much bigger than those of diploids (92×50 μm on average for the triploid and 80×44 μm for the diploid from Japan), and the eggshell of the triploid form is often thickened at the nonoperculated end. The tetraploid form was found to be restricted to Northeast China [13] and their pathogenicity to mammals, including humans, has remained unclear.

From the isozyme studies carried out by Agatsuma et al. [14,15] of *P. westermani* in East and Southeast Asia, it was observed that genetic distances of diploid populations in East Asia (Japan and Taiwan) were different from those in Southeast Asia (Malaysia and the Philippines). The diploid populations from East Asia show relatively high similarity to one another and to the triploid populations, but in Southeast Asia, the diploid populations were genetically distant.

Molecular phylogenies based on the second internal transcribed spacer 2 (ITS2) of the nuclear ribosomal DNA (nrDNA) and cytochrome *c* oxidase subunit I (*cox1*) gene of mitochondrial DNA (mtDNA) have shown that *P. westermani* is partitioned into at least two groups [12,16]. One group comprises members from East Asia (China, Japan, Korea, and Taiwan) and includes both diploid and triploid forms. It was reported that this group exhibits relatively small molecular variation and, therefore, it is postulated that the triploid form arose somewhere in the East Asia group [17]. The second group includes isolates from Southeast Asia (Malaysia, Thailand, and the Philippines) that were genetically distant from one another. These results were in accordance with previous isozyme studies by Agatsuma et al. [14,15]. In 2008, additional *P. westermani* isolates, including isolates from Vietnam, India, and Sri Lanka, were analyzed using sequences of the ITS2 region and *cox1* gene [18], and the resulting phylogenetic trees revealed three groupings of *P. westermani* isolates originating in each of East, Southeast, and South Asia. This grouping seems to be in good agreement with the geographical distributions of *P. westermani* examined to date. In addition, according to Blair et al. [19], phylogenetic analyses of the ITS2 region and *cox1* gene also showed a close relationship between *P. westermani* and *P. siamensis*. All evidence from cytological as well as molecular studies have, therefore, indicated that *P. westermani* consists of a complex of cryptic/sibling species.

Sugiyama et al. [20] found two forms of *P. westermani* metacercariae in a single crab species during intensive field surveys in southern Thailand. The metacercariae were similar in morphology but were quite different in size from each other. The large metacercarial form was referred to as *P. westermani*, and the small form as *P. westermani*-like. In an attempt to obtain adult *P. westermani* and *P. westermani*-like, experimental infections to cats were carried out. Results indicated that the morphological features of *P. westermani*-like were fundamentally identical to those of *P. westermani* (diploid type) at the adult stage. However, the susceptibility of feline hosts to *P. westermani*-like was found to be different from that of *P. westermani*. *P. westermani*-like in the juvenile form were lodged predominantly in the liver, while some matured into adults in the lungs or pleural cavity. In contrast, adult or at least preadult *P. westermani* were detected only from the pleural cavity or lungs. Phylogenetic relationships based on combined sequence data of the ITS2 region and *cox1* gene showed that *P. westermani*-like should be placed as a sister group of *P. westermani* in Southeast and East Asia groups [21]. Taken together, data on the susceptibility and molecular analyses suggest that *P. westermani*-like could be regarded as another form within *P. westermani* species complex.

39.1.1.2 *Paragonimus skrjabini* (Chen, 1959) and *Paragonimus miyazakii* (Kamo et al., 1961)

The first reported specimens of *P. skrjabini* were isolated from the lungs of a viverrid in China. The adult fluke of *P. skrjabini* is long, slender, and spindle-shaped, and its surface is covered with singly spaced cuticular spines. The ventral sucker is slightly larger than the oral sucker and is located anteriorly at about one-third of the length of the body. The ovary and testes are delicately branched and rather small relative to the size of the body. The metacercariae are spherical, with a diameter of 400–450 μm and are enveloped with outer and inner cyst walls. This species occurs in various parts of China as well as in Thailand and the northeastern part of India [4,9,22].

The first reported adult specimens of *P. miyazakii* were isolated from a cat experimentally inoculated with metacercariae. After its discovery, it was confirmed that adults of *P. miyazakii* had been isolated from weasels and martens in western Japan [23]. *P. miyazakii* occurs in Japan.

Blair et al. [12] performed a phylogenetic study and determined that *P. skrjabini* from China was very closely related to *P. miyazakii*; for example, their ITS2 sequences were found to be identical. Blair et al. [11] then compared morphometric data with molecular phylogenetic findings to establish the identities of *P. skrjabini* and its related species, including *P. miyazakii*. They found that *P. skrjabini* from eastern China (Fujian Province) alone was phylogenetically very close to *P. miyazakii* from Japan, proposing that both populations should be referred to as the same subspecies as *P. skrjabini miyazakii*. It was also proposed that *P. skrjabini* populations from other parts of China should be named *P. skrjabini skrjabini*. They also regarded other related species as synonyms (e.g., *Paragonimus szechuanensis*) or cryptic/sibling species (e.g., *Paragonimus hokuoensis*) of *P. skrjabini* and concluded that *P. skrjabini* represents a separate species complex [11]. In this chapter, however, *P. miyazakii* from Japan is not referred to as *P. skrjabini miyazakii* but as *P. miyazakii* hereafter.

P. skrjabini was sometimes designated as *Pagumogonimus skrjabini* in old Chinese literature. In addition, the genus *Euparagonimus* was established for the lung fluke species *Euparagonimus cenocopiosus*. These two additional genera (*Pagumogonimus* and *Euparagonimus*) were created on the basis of adult characters. Recent molecular studies, however, indicated that these two additional genera were artificial and thus untenable [10].

39.1.1.3 *Paragonimus heterotremus* (Chen and Hsia, 1964)

This species occurs from south China to northeast India through Southeast Asian countries such as Vietnam, Laos, and Thailand [9]. The distinctive morphological feature of *P. heterotremus* at the adult stage is the large oral sucker that is more than twice as wide as the ventral one; the specific epithet of this lung fluke comes from that morphological characteristic. In most *Paragonimus* spp., that is, species other than *P. heterotremus* and *Paragonimus africanus* (see below), the oral and ventral suckers are similar in size. The ovary and testes are delicately branched and the latter is larger than the former. The cuticular spines are singly spaced. The metacercariae are readily identifiable due to small size, elliptical shape, and thickening of the inner cyst wall at both polar ends [4].

Recently, Waikagul [24] reported a newly described lung fluke species, *Paragonimus pseudoheterotremus*. This species is morphologically very similar to but genetically distinct from *P. heterotremus*, based on nucleotide sequence differences in *cox1* genes [25]. This finding suggests that *P. pseudoheterotremus* is a cryptic/sibling species to *P. heterotremus*.

39.1.1.4 *Paragonimus africanus* (Voelker and Vogel, 1965)

This species is distributed in sub-Saharan western and central African countries such as Cameroon, Nigeria, Equatorial Guinea, and Ivory Coast [9,26]. The adult fluke has an oral sucker that is considerably larger than the ventral one. The ovary and testes are both complexly branched; the testes are relatively larger than the ovary. The egg is large, with an average size of 91 × 49 μm. The metacercariae are spherical in shape with inner and outer cyst walls [4].

39.1.1.5 *Paragonimus uterobilateralis* (Voelker and Vogel, 1965)

This species is also distributed in sub-Saharan western and central African countries such as Cameroon, Nigeria, Liberia, and Gabon [9,26]. In the adult fluke, the oral and ventral suckers are almost the same size. The ovary is delicately branched while the testes are moderately branched and

larger than the ovary. The cuticular spines are singly spaced. The eggs are much smaller than those of *P. africanus*, measuring 69 × 42 μm on average. The metacercariae are enveloped with a thin single cyst wall [4].

39.1.1.6 *Paragonimus mexicanus* (Miyazaki and Ishii, 1968)

This species is widespread in Central and South American countries from Mexico through Peru and is the only known species causing human paragonimiasis in those areas [9]. The adult fluke possesses profusely divided ovary and testes. The oral sucker is moderately larger than the ventral one, and egg size averages 79 × 48 μm with the surface of the shell showing marked undulation; eggshells of other species listed here have a smooth surface. The metacercariae have no cyst wall and are found free in the tissues of intermediate crab hosts [4].

39.1.1.7 *Paragonimus kellicotti* (Ward, 1908)

This species is found in the United States (east of the Rocky Mountains) and southern Canada [9]. The adult fluke has moderately branched ovary and testes; the former usually smaller than the latter. The oral sucker is slightly smaller than the ventral sucker. Cuticular spines are singly spaced. The egg is large, with an average size of 91 × 57 μm. The metacercariae are spherical in shape with inner and outer cyst walls and are exclusively parasitic in the heart of freshwater crayfish [7].

39.1.2 Biology and Pathogenesis

To complete the life cycle, *Paragonimus* spp. require three hosts [4]. Carnivorous or omnivorous animals and humans are the definitive hosts, freshwater snails are the first intermediate hosts, and freshwater crabs, and occasionally crayfish, are the second intermediate hosts. Adult worms inhabit worm cysts formed in the lungs of definitive hosts. Eggs laid by the adult worms become mixed with sputum, and if sputum is ingested, eggs may be excreted from the hosts to the external environment. In water, the eggs develop further, and miracidia hatch and enter into the first intermediate snail hosts, where they replicate asexually through stages of sporocysts and two generations of rediae and finally produce large numbers of cercariae. These cercariae infect the second intermediate crab or crayfish hosts, and become encysted as metacercariae in the gills, heart, midgut gland, genital organs, or muscle tissues. When the infected second intermediate hosts are eaten by an appropriate definitive host, the larvae excyst from metacercariae in the small intestine and enter through the intestinal wall into the peritoneal cavity and tissue. The growing larvae migrate through the diaphragm into the pleural cavity. Generally, the larvae invade the lung parenchyma after pairing with potential partners despite being hermaphroditic, grow to adult stage in a worm cyst and start laying eggs.

When an infected second intermediate host is ingested by an inappropriate host, larvae may not migrate into the thoracic cavity and inhabit the lung parenchyma, but instead remain in an immature stage, as in the case of *P. skrjabini* infection in humans [27]. Similarly, *P. westermani* larvae in the wild boar migrate to body muscles from the peritoneal cavity and remain there in a larval stage for a long period; these paratenic hosts are now becoming the major source of human infection in Japan [28].

In paragonimiasis, pathological lesions are due to migration of the worms through the tissue, toxic metabolites produced by the growing larvae, the presence of adult worms and eggs and the host's immune response leading to the formation of inflammatory granuloma and encapsulation in the lungs [2].

39.1.3 Clinical Features

Most *Paragonimus* infections are asymptomatic due to the mildness of infection, and spontaneous resolution is common, as demonstrated by high rates of immunologically positive cases in endemic areas [29]. The majority of symptomatic patients develop respiratory symptoms, while others develop symptoms related to the affected organ or tissue. Clinically, paragonimiasis can be classified into three main forms [30,31]: pulmonary, extrapulmonary, and pleuroplumonary.

39.1.3.1 Pulmonary Paragonimiasis

Pulmonary paragonimiasis is the most common form of paragonimiasis [31]. The major presenting symptoms include cough, chest pain, and expectoration of blood-stained or rusty brown sputum, or recurrent hemoptysis, which appear after an incubation period of 2–4 months. Hemoptysis is often increased on physical exertion, and may be severe enough to require hospitalization with blood transfusion.

The common associated symptoms are difficulty in breathing, fever, headache, and weakness, and the more uncommon symptoms are weight loss and anemia. The clinical and radiological features of pulmonary paragonimiasis are indistinguishable from pulmonary tuberculosis, so pulmonary paragonimiasis cases are typically first diagnosed as smear negative pulmonary tuberculosis [1,30,32–34]. Similarly, it is difficult to differentiate pulmonary paragonimiasis from lung cancer [35,36].

39.1.3.2 Extrapulmonary Paragonimiasis

Extrapulmonary paragonimiasis is usually associated with past or present pulmonary infection and is further classified by infestation site.

39.1.3.2.1 Pleural Paragonimiasis

This form, which presents as pleuritis or pleural effusion with or without pneumothorax, occurs in 17% of extrapulmonary paragonimiasis cases [31]. Pleural effusion is usually bilateral and is more common in children. The associated symptoms are chest pain, breathlessness, a cough, and sometimes fever. *Paragonimus* eggs are rarely detected in the pleural fluid aspirate; however, high eosinophil count, low pH, high protein level, low glucose level (<10 mg) and high lactate dehydrogenase (more than or equal to 1000 IU/L) are consistent with pleural paragonimiasis [37,38].

39.1.3.2.2 Cutaneous Paragonimiasis

This form accounts for about 16% of extrapulmonary paragonimiasis cases [31]. Aberrant parasites lodge in the subcutaneous tissue and form migratory subcutaneous nodules. The nodules initially appear on the chest or abdominal wall and then gradually migrate to the inguinal region, genitalia, and lower limbs. The nodules are characteristically mobile, nontender, firm in consistency, and measure 2–5 cm in diameter. Rarely, nodules remain at the location without further migration [39].

39.1.3.2.3 Cardiovascular Paragonimiasis

This is one of the rare but serious conditions in which pulmonary paragonimiasis may be associated with pericarditis, myocarditis, and congestive heart failure, especially in young children. The patient may die of congestive heart failure in spite of praziquantel therapy [40].

39.1.3.2.4 Abdominal Paragonimiasis

Gastrointestinal symptoms develop in most of the patients during the early period of the infection due to the growing larval flukes entering through the intestinal wall into the peritoneal cavity. Abdominal distension, multiple intra-abdominal nodules, hepatomegaly, and peritonitis may also occur due to migration of larval flukes, which probably mature into gravid adults, release eggs and then die [41].

39.1.3.2.5 Neuroparagonimiasis

Cerebral paragonimiasis is the most serious form of paragonimiasis commonly seen in younger age groups [42]. Clinical symptoms include epileptic fits, headache, nausea, vomiting, visual disturbances, blindness, aphasia, and hemiplegia or generalized muscular paresis [43]. Cerebral paragonimiasis can be difficult to differentiate from cerebral tuberculoma, and brain diseases caused by other parasites, such as *Schistosoma japonicum*, *Gnathostoma spinigerum*, and *Angiostrongylus cantonensis* [44,45]. Diagnosis can be made by serological tests and/or imaging techniques such as x-ray, computerized tomography (CT) scan and magnetic resonance imaging (MRI) [46,47].

39.1.3.2.6 Urinary and Ocular Types

Rarely, renal infection causing hematuria, painful scrotal swelling and ocular infection have been reported [27].

39.1.3.3 Pleuroplumonary Paragonimiasis

In about 6% of cases, infection occurs in both the pleural cavity and the lungs [31]. In this form, symptoms of pleural effusion such as pleuritic chest pain and dyspnea are associated with cough, expectoration of blood streaked sputum or recurrent hemoptysis. *Paragonimus* eggs are frequently present in the sputum smears.

39.1.3.4 Differential Diagnosis Based on Clinical Symptoms

The most important clinical symptom of pulmonary paragonimiasis is the expectoration of blood-stained sputum or recurrent hemoptysis. Differential diagnosis of infectious causes of hemoptysis should include pulmonary paragonimiasis, tuberculosis, bronchitis, bronchiectasis, pneumonia, hydatid disease, amebiasis, schistosomiasis, aspergillosis, and histoplasmosis. However, most standard text books hardly mention pulmonary paragonimiasis, and this may be the reason why clinicians usually fail to diagnose cases presenting with hemoptysis as pulmonary paragonimiasis. Careful history taking and thorough laboratory investigations are the preliminary steps in elucidating the cause of hemoptysis, although the cause may not be determined in about 50% of the cases. In areas with high incidences of paragonimiasis, pulmonary paragonimiasis should always be considered for patients presenting with hemoptysis who have a history of consuming crabs or crayfish [31,48].

39.1.4 Diagnosis

Conventional methods for the diagnosis of *Paragonimus* infections usually require detection of eggs discharged in sputum or stool samples. However, eggs are not always observed in clinical specimens, especially during the prepatent period of infection and in extrapulmonary paragonimiasis. To overcome the limitations of conventional methods in detecting eggs, immunological tests such as enzyme-linked immunosorbent assay (ELISA) have been applied to achieve a quick but adjunct diagnosis.

39.1.4.1 Conventional Techniques

39.1.4.1.1 Microscopic Examination of Clinical Samples for Paragonimus Eggs

A definitive diagnosis of paragonimiasis is usually made by finding characteristic golden brown, ellipsoidal or oval, and operculated *Paragonimus* eggs in the sputum or feces but rarely in the pleural fluid. Direct microscopic examination of at least three early-morning sputum samples is recommended for the detection of *Paragonimus* eggs in (pleuro)pulmonary paragonimiasis.

Similarly, three to four fresh or formalin-preserved fecal samples should be examined for the detection of *Paragonimus* eggs either by direct wet mount cover slip preparation or after AMS III concentration techniques [49] or formol-ether sedimentation technique (the modified method is recommended) [50] for a definitive diagnosis. Examination of stool samples is especially important in suspected, but apparently sputum-negative cases and in young children who usually do not expectorate but swallow sputum [30].

Charcot Leyden crystals (CLC) are usually present in the sputum of paragonimiasis cases, even when *Paragonimus* eggs are not detectable. Therefore, the presence of CLC in the sputum in a clinically and epidemiologically suspected case is a strong indication of pulmonary paragonimiasis [30].

39.1.4.1.2 Hematology Investigation

Paragonimiasis, especially in children, is characterized by marked eosinophilia with absolute eosinophil counts ranging from 650 to 4000/mm^3 and increased erythrocyte sedimentation rate. Therefore, elevated eosinophil count may strongly indicate diagnosis of paragonimiasis

in a clinically and epidemiologically suspected case. Many cases of paragonimiasis present with leucocytosis characterized by a total white blood cell count varying from 10,200 to 16,350/mm^3 and mild lymphocytosis [30,38]. In contrast, hemoglobin values remain within normal limits despite frequent expectoration of blood-stained sputum or recurrent hemoptysis in most patients.

39.1.4.1.3 X-Ray and Other Imaging Techniques

The common abnormal findings in the chest roentgenograms are ill-defined patchy consolidations 5–60 mm in size (63%), pleural thickening or blunting of costophrenic angle (53%), nodular densities 1–4 cm in diameter (13%), cavities (11%), and pleural effusion that is usually bilateral (60%) [32]. Chest roentgenograms may be normal (15%), even in symptomatic patients with or without detectable *Paragonimus* eggs in sputum specimens [32]. CT and MRI are better techniques for visualization of the lesions in the lungs and lungs/brain, respectively [46,47,51].

39.1.4.1.4 Immunological Techniques

Immunological techniques play an important role in diagnosis during the prepatent period of infection and in extrapulmonary paragonimiasis in which eggs are not detectable in typical clinical specimens. Tests based on various immunological techniques have been used: intradermal (ID) test, complement fixation test (CFT), immunodiffusion test, indirect hemagglutination (IHA) test, ELISA, and immunoblotting.

39.1.4.1.5 ID Test

The ID test is simple, easy to perform, inexpensive, rapid, and highly sensitive; it was widely and effectively used over the past several decades in Japan [2]. The test has been used in countries such as India [29] and Laos [52] for mass screening in epidemiological surveillance of paragonimiasis as well as laboratory diagnosis. It is valuable in distinguishing pulmonary paragonimiasis from pulmonary tuberculosis, especially in areas where these are coendemic. The test is performed by intradermally inoculating of the test antigen on the volar aspect of the forearm and observing wheal formation diameter 15 min after inoculation. The major disadvantage of this test is the inability to distinguish between past and current infection as a positive result may remain as long as 10–20 years after successful treatment or spontaneous recovery [2]. It is also possible that cross reaction with other trematodiasis like schistosomiasis and clonorchiasis will produce a positive result if crude antigen is used, but this can be avoided by using purified and fractionated antigen; the sensitivity and specificity of the ID test can be up to 100% with the purified and fractionated antigen [53].

39.1.4.1.6 Complement Fixation Test

The CFT is a highly specific test used to diagnose active infection and to confirm ID positive cases. Because the test result becomes negative within 3–9 months after successful treatment, it can be used for therapeutic evaluation [54]. This test is not suitable for routine use owing to its lower sensitivity, long sampling-to-result time, labor intensiveness, and that it requires experienced technicians.

39.1.4.1.7 Immunodiffusion Test

Biguet et al. [55] first developed the immunodiffusion method for the diagnosis of paragonimiasis. The double-diffusion technique (Ouchterlony's method), immunoelectrophoresis, and counter-current electrophoresis are highly sensitive and specific tests and can be used for species differentiation by demonstration of specific precipitation bands [56–58].

39.1.4.1.8 IHA Test

The IHA test is another simple, rapid, and highly sensitive test used in the diagnosis of human paragonimiasis [59].

39.1.4.1.9 Enzyme-Linked Immunosorbent Assay

The first report of the application of ELISA in the diagnosis of paragonimiasis in Thailand was made in 1981 [60]. ELISA is now most widely used as serological tests of choice for their high sensitivity and specificity [61]. The tests are also applicable to mass screening [62]. ELISA tests based on different techniques and with different antigen preparations have been developed; other ELISA techniques include sandwich ELISA, which uses a monoclonal antibodies-based antigen detection assay [63] and multiple dot-ELISA, which is now commonly used in Japan for diagnostic purposes [64]. Different antigen preparations include F1 antigen, which attains 100% sensitivity and specificity in *P. heterotremus* infection [65]. ELISA test using excretory and secretory (ES) products of *Paragonimus* as the antigen also affords high sensitivity and specificity in the diagnosis of paragonimiasis [66]. Generally, although the ELISA has been used to detect parasite-specific IgG antibodies, the IgM ELISA has proven to be useful for the diagnosis of infection in its early stages [67] and IgE-ELISA was suggested to be useful in evaluating chemotherapy for paragonimiasis due to the short half-life of IgE antibodies [68]. An ELISA inhibition test has been established to differentiate infection-causing lung fluke species [36]. The test serum was incubated with either serially diluted *P. westermani* or *P. miyazakii* antigen, and the set of serum samples was used to measure the antibody titers against the respective antigens by ELISA. The reactivity of the test serum was inhibited more effectively after incubation with the homologous (causative species) antigen. The disadvantages of ELISA tests are that they are time consuming and require expensive equipment and experienced technicians.

39.1.4.1.10 Immunoblotting

Researchers have attempted to use sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting techniques to characterize the antigenic substances that react with sera of infected patients and test animals. Using this technique, Sugiyama et al. [69] determined that the 27-kDa protein(s) in the adult fluke antigen is applicable for diagnosis of paragonimiasis westermani in not only definitive hosts but also in paratenic hosts. They also determined that the antigenic material is located in the gut of both adult and

immature flukes by the immunoperoxidase staining technique [69], suggesting that the antigen(s) are probably ES products. The reactive band of 31.5 kDa in the immunoblot assay was described as a specific antigenic marker for *P. heterotremus* infection, which can be used for the serodiagnosis of infected patients [70] and also to evaluate the effect of praziquantel therapy [71]. The differential diagnosis between *P. heterotremus* and *P. westermani* infections was made using adult *P. heterotremus* extracts [72]; a 35-kDa molecule was found to be *P. heterotremus*-specific. Generally, though immunoblotting analyses detect parasite-specific IgG antibodies, IgE immunoblot was carried out and proved to reduce cross-reactions with other trematode infections [73].

39.1.4.1.11 Rapid Test

Recently, the dot-immunogold filtration assay (DIGFA) kit was developed in China for the detection of anti-*P. westermani* antibodies. The kit is based on the principle of a membrane-based flow-through immunoassay technique. Anti-human IgG conjugated with gold-colloids are applied after the test sera to the kit and the results are visualized within 10 min. The kit was prepared using *P. westermani* antigen and the sensitivity and specificity of the kit were reported to be up to 99% and 92%, respectively [74]. The kit was also found to be applicable in Japan to detect serum antibodies in patients infected with either *P. westermani* or *P. miyazakii* [75].

39.1.4.2 Molecular Techniques

The morphological characteristics of adults and metacercariae specimens, and sometimes eggs, are used to identify and discriminate the *Paragonimus* spp. (see Section 39.1.1). However, it is not always possible to make an identification of the causative species based only on the morphological characteristics of specimens available in clinical samples. In that case, molecular-based diagnostic techniques including PCR and DNA sequencing have been applied [76,77]. Other potential techniques, such as restriction fragment-length polymorphism (RFLP) analysis, PCR-RFLP, multiplex PCR, random amplified polymorphic DNA (RAPD) and DNA hybridization, have also been explored for the identification and discrimination of *Paragonimus* spp. using specimens at various life cycle stages as well as having damaged, incomplete, or indistinguishable morphological characteristics [6]. All these techniques have also been utilized for the genetic characterization and/or phylogenetic analysis of paragonimiasis causative agents. The data obtained from these investigations can be further applied to paragonimiasis control programs.

39.1.4.2.1 PCR and DNA Sequencing

In 2000, Chang et al. [77] amplified and sequenced the ITS2 region from eggs recovered from sputum of a Chinese patient with paragonimiasis. Sequence alignment showed 100% similarity with a sequence from *P. westermani* eggs obtained from an experimentally infected dog, indicating the causative agent to be *P. westermani*. A similar method was applied by Singh et al. [48,76] to determine the etiological agent of human paragonimiasis in India as *P. heterotremus*. In Vietnam, Le et al. [78] used *Paragonimus* DNA from different life cycle stages, including eggs, miracidia and adults recovered from a human host. Their phylogenetic trees, based on ITS2 region and *cox1* gene sequences, revealed a distinct group of *P. heterotremus* from Vietnam, Thailand, and China. These findings also demonstrated that, regardless of life cycle stage of the specimens used, molecular techniques were accurate and rapid in identifying this parasite as *P. heterotremus*. In addition, PCR methods to detect copro-DNA (genomic DNA derived from *Paragonimus* eggs in feces) have been developed. Using fecal samples, Intapan et al. [79] and Nkouawa et al. [80] demonstrated that the approaches were effective in diagnosing *P. heterotremus* and *P. africanus* infections in cats and humans, respectively.

39.1.4.2.2 Restriction Fragment-Length Polymorphism

Agatsuma et al. [81] examined genetic differences between the Japanese diploid and triploid *P. westermani*. RFLP analysis of the entire mitochondrial genome using *Pst*I endonuclease showed that all diploids produced two bands while only one band was generated by triploids. Blair et al. [12] mentioned that the parthenogenetic triploid form was more pathogenic than the diploid form, making it possible to use molecular techniques to distinguish these two medically important forms.

39.1.4.2.3 PCR-RFLP

The prevalence of lung fluke metacercariae, which are pathogens that cause human paragonimiasis, can be investigated using PCR-based techniques and DNA sequencing. The metacercariae of *P. westermani* and *P. miyazakii*, which are morphologically quite similar, were discriminated based on the ITS2 region (520 bp) using PCR-RFLP and PCR amplification with species-specific primers. For PCR-RFLP, restriction enzymes *Sna*BI and *Bss*SI were used; the former cleaved only the ITS2-amplified product from *P. westermani*, producing two fragments of about 420 and 100 bp, and the latter only digested *P. miyazakii* PCR products and generated two fragments of about 300 and 220 bp. Another technique, using either a *P. westermani*-specific primer (PwR1) or a *P. miyazakii*-specific primer (PmR1), yielded PCR products of about 420 bp only from the respective species when applied with a consensus forward primer (3S) [82].

39.1.4.2.4 Multiplex PCR

Molecular methods established to differentiate among species have relied mainly on nucleotide differences in the ITS2 region. Sugiyama et al. [83] developed a multiplex PCR method which used interspecies conserved and species-specific primers designed from the sequences of the ITS2 region, and which allowed species discrimination of individual metacercariae in a single-tube reaction. The newly designed forward primers, PwF1 and PmF1, were used in combination with the consensus reverse primer, A28, to obtain specific PCR products from *P. westermani* and *P.*

miyazakii, respectively. Although about 520 bp PCR products were obtained from both species, each product had a distinctive band, that is, about 140 bp for *P. westermani* and about 300 bp for *P. miyazakii*.

39.1.4.2.5 Random Amplified Polymorphic DNA

Intapan et al. [84] reported the use of another PCR-based technique, known as RAPD, to discriminate among five species of *Paragonimus* in Thailand. RAPD patterns obtained from amplification with an arbitrary primer, P2, showed that *P. heterotremus*, *P. harinasutai*, *P. siamensis*, *P. bangkokensis* and *P. westermani* were genetically distinct. A dendrogram constructed from the observed genetic polymorphisms revealed a close relationship between *P. heterotremus* and *P. harinasutai*.

39.1.4.2.6 DNA Hybridization

In 1997, Maleewong et al. [85] established a DNA hybridization technique for the detection of *P. heterotremus* DNA in fecal samples of experimentally infected cats. A radioactively labeled probe utilizing a highly repetitive DNA fragment (ca. 1500 bp; probably a portion of a retrotransposon [8]) derived from a *P. heterotremus* genomic DNA library could detect as few as two *P. heterotremus* eggs. This method has 100% specificity and sensitivity.

39.2 METHODS

Sample collection and preparation for molecular methods for identifying and discriminating *Paragonimus* spp. are described below. Detection procedures such as PCR, PCR-RFLP analysis, and cycle sequencing are also described.

39.2.1 Sample Preparation

39.2.1.1 Eggs

Detection and collection procedures of eggs from the sputa and/or feces of patients and definitive mammalian hosts are described above (see Section 39.1.4.1.1). If the sputum sample contains much mucus, the sample should be sedimented before isolating eggs. Briefly, digest the sputum with five volumes of 5% sodium hydroxide for 10 min and centrifuge for 5 min at 2000 × *g*. This procedure, as well as AMS III concentration or formol-ether sedimentation techniques, does not affect the integrity of eggs for further molecular examination.

A method has been developed to extract DNA from a small number of *P. westermani* eggs using proteinase K (Qiagen) and NP-40 (e.g., Tergitol-type NP-40®, Sigma-Aldrich Co.) for amplification by PCR [77]. More effective methods have also been applied in order to extract DNA from individual eggs [48,76]; a method using proteinase K and SDS was originally developed for extracting DNA from metacercariae [86]. Prior to the start of the extraction by these methods, the additional step of puncturing the shell of the egg with a dissecting needle is recommended [87]. This step gives the extraction solution access to the vitelline cells and oocytes inside the egg.

39.2.1.1.1 Reagents: Proteinase K-SDS solution (1 mL) [86]

1. 30 µL of 5 M NaCl.
2. 10 µL of 1 M Tris-Cl pH 8.0.
3. 20 µL of 0.5 M EDTA.
4. 5 µL of 20% SDS.
5. 10 µL of proteinase K (20 mg/mL).
6. 925 µL of distilled water to adjust the total volume of the working solution to 1 mL.

39.2.1.1.2 Procedure

1. Place several eggs on a concave slide (i.e., a glass microscope slide having a concave cell in the center of the slide) filled with distilled water.
2. Wash the eggs with several changes of distilled water using a micropipette under a dissecting microscope.
3. Puncture the shell of each egg with a dissecting needle.
4. Transfer each punctured egg in a small amount of water, for example, 1 µL, into each 1.5 mL microcentrifuge tube or 0.6 mL thin-walled microcentrifuge tube.
5. Add 7 µL of proteinase K-SDS solution to each tube and incubate the tubes at 60°C for 1 h to overnight (until the tissue in each tube becomes solubilized).
6. Add the following reagents to each tube and then hold the tube at −80°C for 10 min for ethanol precipitation.
   a. 0.8 µL of 3 M sodium acetate (pH 5.2).
   b. 20 µL of absolute ethanol.
7. Centrifuge the tubes at 4°C at 8000 × *g* for 10 min to pellet the extracted DNA; discard the supernatant.
8. Rinse the pellets with 0.1 mL of 70% ethanol.
9. Centrifuge for 10 min with the same conditions; discard the supernatant.
10. Place the tube at 60°C for 1 min to evaporate the remaining ethanol.
11. Add an appropriate amount of Tris-EDTA buffer, for example, 50 µL, to each tube and heat the tubes at 95°C for 10 min to inhibit proteinase K activity.
12. Determine the DNA concentration spectrophotometerically and proceed to PCR amplification.

39.2.1.2 Adult and Immature Flukes

Occasionally, adult worms are expectorated in the sputum [88] or recovered from the cyst in tissue removed by surgery [89] or at necropsy [90]. Immature (without eggs) or adult (with eggs) worms are isolated or found in the biopsy material of subcutaneous nodules in cases of cutaneous paragonimiasis [91]. Adult flukes are also isolated from worm cysts formed in the lungs of definitive mammalian hosts, naturally or experimentally infected with *Paragonimus* spp.; they are used for positive identification of species by molecular methods.

The above-mentioned proteinase K-SDS method is also applicable to the extraction of DNA from immature flukes

and small fragments of adult flukes. DNA is also extracted from adult and immature flukes using commercially available kits, for example, DNeasy Blood and Tissue Kit® (Qiagen) or NucleoSpin Tissue Kit® (Macherey-Nagel). The E.Z.N.A. Mollusk DNA Isolation Kit® (Omega Biotek) is also recommended for extracting genomic DNA from lung flukes at different life cycle stages, even for specimens frozen or preserved in alcohol [6]. This kit is efficient for recovering genomic DNA from other invertebrates, including flatworms which have tissues rich in mucopolysaccharide, but the kit is disadvantageous in that it is necessary to prepare organic solvents other than ethanol, for example, chloroform and isoamyl alcohol.

39.2.1.3 Metacercariae

To gather epidemiological data from the source regions, *Paragonimus* metacercariae are collected by microscopic examination of crabs or crayfish, the second intermediate hosts, which also act as the infection sources to humans and other mammals. The preferred lodging sites of metacercariae in these crustacean hosts have been demonstrated in some *Paragonimus* spp. to be as follows: the body (cephalothorax) muscles for *P. westermani* (the diploid form in Japan) [92], the heart for *P. miyazakii* [93], and the leg (appendage) muscles for *P. heterotremus* (in Thailand) [94]. However, this information is limited to only a few species, and all the internal organs (gills, hearts, midgut gland, and genital organs) and muscle tissues (body and legs) are usually examined for each specimen. As the crustacean hosts are poikilothermic animals, they are immobilized by being placed on ice or immersed in chilled water. This facilitates the removal of the carapace and legs from the body by hand without the aid of any instruments. After removing the carapace, the internal organs and body muscles from each crustacean host are separately excised with tweezers, compressed between two glass plates (typically, $10 \times 6 \times 0.2$ (thickness) cm), and examined for metacercariae under a stereoscopic microscope. The leg muscles are also removed with tweezers after breaking the shell with a nipper, and examined in a same manner. When *Paragonimus* metacercariae are identified, the glass plate covering the tissues is carefully removed and metacercariae in the compressed tissues are recovered using teasing needles or tweezers.

To gather general epidemiological information, the crustacean hosts are collected together and crushed in bulk using a kitchen blender or manual meat-mincing machine. Minced tissues are then washed with large volumes of tap water and repeatedly filtered through wire sieves, for example, 1-mm mesh. The washed sediment of the filtrate is examined under a stereoscopic microscope to detect *Paragonimus* metacercariae.

We use artificial gastric juice (acid-pepsin solution) consisting of 1 g of pepsin (e.g., Nacalai Tesque, Inc.; 1:10,000) in 1 L of 0.7% hydrochloric acid to facilitate the digestion of the host tissue [95]. Minced crab tissues are incubated with artificial gastric juice at 37°C for 1–2 h with shaking or stirring. The subsequent digest is filtered through a wire sieve, for example, 1-mm mesh, allowed to sediment, and washed with several changes of tap water followed by observation under a microscope to isolate metacercariae, as described above. Isolated metacercariae should be kept frozen (–20°C or lower) or in a tube with 70% ethanol at room temperature to extract DNA by the above-mentioned method [86].

39.2.2 Detection Procedures

DNA techniques utilizing genetic markers in nrDNA and mtDNA have been employed to resolve taxonomic issues related to several species of the genus *Paragonimus* at various life cycle stages. Blair et al. [11,12] suggested that the ITS2 region of nrDNA is a good interspecies marker while that of the partial *cox1* gene of mtDNA is a good intraspecies marker. The fragments for sequence analyses were generated by PCR of genomic DNA using universal primers reported previously or designed originally by researchers themselves. After successful PCR amplification, the amplified products were visualized as a band with a particular size on an agarose gel. The PCR products were further sequenced and analyzed by the procedures including a PCR-RFLP method.

For PCR amplification of the ITS2 region of *Paragonimus* spp., the following primer pair was used [12,96]:

1. An interspecies-conserved forward primer (3S): 5′-GGTACCGGTGGATCACTCGGCTCGTG-3′.
2. An interspecies-conserved reverse primer (A28): 5′-GGGATCCTGGTTAGTTTCTTTTCCTC CGC-3′.

For PCR amplification of the partial *cox1* gene of *Paragonimus* spp., the following primer pair was used [96]:

3. An interspecies-conserved forward primer (JB3): 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′.
4. An interspecies-conserved reverse primer (JB4.5): 5′-TAAAGAAAGAACATAATGAAAATG-3′.

39.2.2.1 Reagents for PCR

Reagents for PCR mixtures and the volume of each reagent required for a single reaction (50 μL) are prepared according to the manufacturer's instruction for TaKaRa EX *Taq*® (Takara Bio Inc.) as follows:

1. 0.5 μL of DNA polymerase, for example, TaKaRa EX *Taq* (1.25 U).
2. 1 μL of dNTPs (supplied by the manufacturer in 10 mM stocks).
3. 5 μL of 10× buffer (containing 100 mM Tris-HCl pH 8.3, 500 mM KCl, and 15 mM $MgCl_2$).
4. 1.25 μL of forward primer (20 μM stock).
5. 1.25 μL of a reverse primer (20 μM stock).
6. 1–10 μL of template DNA (prepared at 10–100 ng/μL).
7. 31–40 μL of distilled water to make each mixture volume up to 50 μL.

39.2.2.2 PCR Amplification and Visualization of the Amplified Products

PCR is carried out on a thermal cycler (e.g., TaKaRa PCR Thermal Cycler Dice Standard®) with 35 cycles of 98°C for 5 s, 55°C for 30 s, and 72°C for 60 s. A final extension is carried out at 72°C for 10 min.

The PCR products are separated by electrophoresis on 1.0% agarose gels in Tris-borate-EDTA buffer (TBE buffer) and visualized by illumination with short-wave ultraviolet light after ethidium bromide staining.

39.2.2.3 PCR-RFLP Analysis

For species identification and discrimination by PCR-RFLP, the restriction enzymes are selected on the basis of the theoretical restriction maps generated from the sequences of the target lung fluke species. The PCR products are digested with the selected restriction enzyme(s) according to the manufacturer's recommendations. The digested samples are then separated by electrophoresis on agarose gels and visualized as described above.

39.2.2.4 Cycle Sequencing

The PCR products are purified by removing primers from the PCR reactions using a commercially available kit, for example, NucleoSpin Extract II® (Macherey-Nagel). The purified products are sequenced using a BigDye Terminator Cycle Sequencing Kit® (Life Technologies) on an automated sequencer (e.g., ABI3100®, Life Technologies).

39.3 CONCLUSIONS AND PERSPECTIVES

Paragonimiasis caused by the lung fluke belonging to the genus *Paragonimus* is an important foodborne helminthic zoonosis; almost 300 million people around the world are estimated to be at risk for infection. The common mode of infection has been the consumption of raw or undercooked second intermediate hosts (freshwater crabs or crayfish) or raw or improperly cooked meat of paratenic hosts (e.g., wild boar). Pulmonary paragonimiasis is difficult to differentiate from pulmonary tuberculosis due to similarities in their clinical and radiological manifestations. Therefore, patients presenting with respiratory symptoms (e.g., hemoptysis and chronic cough) should also be investigated for paragonimiasis before prescribing antitubercular drugs for smear-negative pulmonary tuberculosis or multidrug-resistant tuberculosis. This will have a significant impact on the tuberculosis control program, especially in the areas where both diseases are sympatrically prevalent. Once diagnosed, paragonimiasis can be effectively treated with praziquantel [97].

The diagnosis of paragonimiasis is primarily based on the detection of eggs in sputa or feces. However, eggs are not always observed in clinical specimens, especially during the prepatent period of infection and in extrapulmonary paragonimiasis. To overcome these limitations, various serological methods including highly sensitive and specific tests have been developed for laboratory diagnosis of paragonimiasis. Molecular techniques have been explored for the identification of *Paragonimus* spp. at various life cycle stages, not limited to eggs but also adult and immature flukes, and metacercariae. Moreover, molecular approaches also have the potential to be used for species identification of specimens having damaged, incomplete, or indistinguishable morphological characteristics. The techniques used for these aims include PCR and DNA sequencing, PCR-RFLP, multiplex PCR, RAPD, and DNA hybridization.

Molecular techniques have also been applied for taxonomic studies. In this context, *P. westermani* has been extensively examined because this lung fluke species shows great diversity in geographical distribution, host susceptibility, pathogenicity, and even morphology, especially in metacercarial and egg stages. Molecular phylogenetic analyses consequently have revealed that the phylogenetic tree of *P. westermani* has three single clades, each comprising isolates from three distinct regions. This grouping is in good agreement with not only the geographical distribution, but also host susceptibility and cytological findings. Phylogenetic analyses also showed that *P. siamensis*, a species discriminated from *P. westermani* by morphology, has a close genetic relationship with *P. westermani*, indicating that *P. westermani* consists of species complex. In addition, *P. skrjabini* represents a separate species complex. Based on recent advances in molecular studies of *Paragonimus* spp., the concept of the species complex is now and will become more widely accepted [8,98]. To resolve the discrepancy between systematics in morphology and by phylogenetic analysis, it is suggested that the morphological and morphometric parameters traditionally applied for the speciations of *Paragonimus* spp. should be reevaluated.

The strategy for the prevention and control of paragonimiasis should include: (i) Information, Education and Communication (IEC) for the public with emphasis on safe habits of eating and the cooking of freshwater crabs, crayfish, and paratenic hosts, which transmit the infection, and the effects of the paragonimiasis on human health. (ii) IEC and adequate training for health care personnel about the epidemiology, life cycle, clinical features, prevention at all three levels (primary, secondary, and tertiary) and treatment of cases.

However experiences have shown little success as the eating traditions and cultural practices of people in endemic areas are difficult to change.

REFERENCES

1. Toscano, C. et al. Paragonimiasis and tuberculosis, diagnostic confusion: A review of the literature. *Trop. Dis. Bull.* 92, R1, 1995.
2. Yokogawa, M. Paragonimiasis. In: Hillyer, G.V., and Hopla, C.E. (eds.), *CRC Handbook Series in Zoonoses*, Vol. III. CRC Press, Inc., Boca Raton, p. 123, 1982.
3. Miyazaki, I. Paragonimiasis. In: Hillyer, G.V., and Hopla, C.E. (eds.), *CRC Handbook Series in Zoonoses*, Vol. III. CRC Press, Inc., Boca Raton, p. 143, 1982.
4. Miyazaki, I. Paragonimiasis. In: Miyazaki, I. (ed.), *An illustrated Book of Helminthic Zoonoses*. International Medical Foundation of Japan, Tokyo, p. 76, 1991.

5. Keiser, J., and Utzinger, J. Emerging foodborne trematodiasis. *Emerg. Infect. Dis.* 11, 1507, 2005.
6. Narain, K., Agatsuma, T., and Blair, D. *Paragonimus*. In: Liu, D.Y. (ed.), *Molecular Detection of Foodborne Pathogens*. Chapter 61. CRC Press, Inc., Boca Raton, p. 827, 2009.
7. Miyazaki, I. Lung flukes in the world–Morphology and life history. In: Sasa, M. (ed.), *A Symposium on Epidemiology of Parasitic Diseases*, International Medical Foundation of Japan, Tokyo, p. 101, 1974.
8. Blair, D., Agatsuma, T., and Wang, W. Paragonimiasis. In: Murrell, K.D., and Fried, B. (eds.), *World Class Parasites: Food-borne Parasitic Zoonoses: Fish and Plant-Borne Parasites*, Vol. 11. Springer, New York, p. 117, 2007.
9. Blair, D., Xu, Z.B., and Agatsuma, T. Paragonimiasis and the genus *Paragonimus*. *Adv. Parasitol.* 42, 113, 1999.
10. Blair, D. et al. A molecular perspective on the genera *Paragonimus* Braun, *Euparagonimus* Chen and *Pagumogonimus* Chen. *J. Helminthol.* 73, 295, 1999.
11. Blair, D. et al. *Paragonimus skrjabini* Chen, 1959 (Digenea: Paragonimidae) and related species in eastern Asia: A combined molecular and morphological approach to identification and taxonomy. *Syst. Parasitol.* 60, 1, 2005.
12. Blair, D. et al. Geographical genetic structure within the human lung fluke, *Paragonimus westermani*, detected from DNA sequences. *Parasitology* 115, 411, 1997.
13. Terasaki, K. et al. Tetraploids of the lung fluke *Paragonimus westermani* found in China. *Parasitol. Res.* 81, 627, 1995.
14. Agatsuma, T., and Habe, S. Electrophoretic studies on enzymes of diploid and triploid *Paragonimus westermani*. *Parasitology* 91, 489, 1985.
15. Agatsuma, T. et al. Genetic differentiation between Malaysian and other Asian *Paragonimus westermani*. *Trop. Biomed.* 10, 45, 1993.
16. Iwagami, M. et al. Molecular phylogeographic studies on *Paragonimus westermani* in Asia. *J. Helminthol.* 74, 315, 2000.
17. Park, G.M., Im, K.I., and Yong, T.S. Phylogenetic relationship of ribosomal ITS2 and mitochondrial COI among diploid and triploid *Paragonimus westermani* isolates. *Korean J. Parasitol.* 41, 47, 2003.
18. Doanh, P.N. et al. Discovery of *Paragonimus westermani* in Vietnam and its molecular phylogenetic status in *P. westermani* complex. *Parasitol. Res.* 104, 1149, 2009.
19. Blair, D. et al. Phylogenetic relationships among the Thai species of *Paragonimus* inferred from DNA sequences. In: Tada, I., Kojima, S., and Tsuji, M. (eds.), *IX International Congress of Parasitology (ICOPA IX)*, Monduzzi Editore, Bologna, p. 643, 1998.
20. Sugiyama, H. et al. New form of *Paragonimus westermani* discovered in Thailand: Morphological characteristics and host susceptibility. *Southeast Asian J. Trop. Med. Public Health* 38(Suppl. 1), 87, 2007.
21. Binchai, S. et al. Molecular systematics of a new form of *Paragonimus westermani* discovered in Thailand, *Southeast Asian J. Trop. Med. Public Health* 38(Suppl. 1), 92, 2007.
22. Singh, S.T., Singh, D.L., and Sugiyama, H. Possible discovery of Chinese lung fluke, *Paragonimus skrjabini* in Manipur, India. *Southeast Asian J. Trop. Med. Public Health* 37(Suppl. 3), 53, 2006.
23. Kamo, H. et al. On the occurrence of a new lung fluke, *Paragonimus miyazakii* n. sp. in Japan (Trematoda: Troglotrematidae). *Yonago Acta Med.* 5, 43, 1961.
24. Waikagul, J. A new species of *Paragonimus* (Trematoda: Troglotrematidae) from a cat infected with metacercariae from mountain crabs *Larnaudia larnaudii*. *J. Parasitol.* 93, 1496, 2007.
25. Thaenkham, U., and Waikagul, J. Molecular phylogenetic relationship of *Paragonimus pseudoheterotremus*. *Southeast Asian J. Trop. Med. Public Health* 39, 217, 2008.
26. Aka, N.A. et al. Human paragonimiasis in Africa. *Ann. Afr. Med.* 7, 153, 2008.
27. Zhong, H.L. et al. Recent progress in studies of *Paragonimus* and paragonimiasis control in China. *Chin. Med. J.* 94, 483, 1981.
28. Uchiyama, F., Morimoto, Y., and Nawa, Y. Re-emergence of paragonimiasis in Kyushu, Japan. *Southeast Asian J. Trop. Med. Public Health* 30, 686, 1999.
29. Singh, T.S. et al. Paragonimiasis in Manipur. *Indian J. Med. Res.* 97, 247, 1993.
30. Singh, T.S., Mutum, S.S., and Razaque, M.A. Pulmonary paragonimiasis: Clinical features, diagnosis and treatment of 39 cases in Manipur. *Trans. R. Soc. Trop. Med. Hyg.* 80, 967, 1986.
31. Singh, T.S., and Sugiyama, H. Paragonimiasis in India: A newly emerging food borne parasitic disease. *Clin. Parasitol.* 19, 95, 2008.
32. Razaque, M.A., Mutum, S.S., and Singh, T.S. Recurrent haemoptysis? Think of paragonimiasis. *Trop. Doct.* 21, 153, 1991.
33. Ogakwu, M., and Nwokolo, C. Radiological findings in pulmonary paragonimiasis as seen in Nigeria: A review based on one hundred cases. *Br. J. Radiol.* 46, 699, 1973.
34. Romeo, D.P., and Pollock, J.J. Pulmonary paragonimiasis: Diagnostic value of pleural fluid analysis. *South. Med. J.* 79, 241, 1986.
35. Watanabe, S. et al. Pulmonary paragonimiasis mimicking lung cancer on FDG-PET imaging. *Anticancer Res.* 23, 3437, 2003.
36. Yoshino, I. et al. Paragonimiasis westermani presenting as an asymptomatic nodular lesion in the lung: Report of a case. *Surg. Today* 28, 108, 1998.
37. Johnson, R.J., and Johnson, J.R. Paragonimiasis in Indochinese refugees. Roentgenographic findings with clinical correlations. *Am. Rev. Respir. Dis.* 128, 534, 1983.
38. Jeon, K. et al. Clinical features of recently diagnosed pulmonary paragonimiasis in Korea. *Chest* 128, 1423, 2005.
39. Chung, H.L., and Tsao, W.C. *Paragonimus westermani* (Szechuan variety) and a new species of lung fluke–*paragonimiasis szechuanensis*, part II. Studies on clinical aspects of *Paragonimiasis szechuanensis*: A new clinical entity. *Chin. Med. J.* 81, 419, 1962.
40. Singh, P.I., Singh, I.P., and Singh, L.B.M. Paragonimiasis: Review of 45 cases. *Indian J. Med. Microbiol.* 10, 243, 1992.
41. Jeong, M.G. et al. Retroperitoneal paragonimiasis: A case of ectopic paragonimiasis presenting as periureteral masses. *J. Comput. Assist. Tomogr.* 23, 696, 1999.
42. Singh, T.S., Khamo, V., and Sugiyama, H. Cerebral paragonimiasis mimicking tuberculoma: First case report in India. *Trop. Parasitol.* 1, 39, 2011.
43. Miyazaki, I., and Nishimura, K. Cerebral paragonimiasis. In: Hornabrook, R.W. (ed.), *Topics on Tropical Neurology*, Contemporary Neurology Series Vol. 12, Chapter 7. F.A. Davis Company, Philadelphia, p. 109, 1975.
44. Hughes, A.J., and Biggs, B.A. Parasitic worms of the central nervous system: An Australian perspective. *Intern. Med. J.* 32, 541, 2002.
45. Jaroonvesama, N. Differential diagnosis of eosinophilic meningitis. *Parasitol. Today* 4, 262, 1988.

46. Chang, K.H. et al. An imaging diagnosis of cerebral paragonimiasis: CT and MR findings and correlation with ELISA antibody test. *J. Korean Radiol. Soc.* 29, 345, 1993.
47. Choo, J.D. et al. Chronic cerebral paragonimiasis combined with aneurismal subarachnoid haemorrhage. *Am. J. Trop. Med. Hyg.* 69, 466, 2003.
48. Singh, T.S. et al. *Paragonimus heterotremus* infection in Nagaland: A new focus of paragonimiasis in India. *Indian J. Med. Microbiol.* 27, 123, 2009.
49. Komiya, Y., and Yokogawa, M. The recovering of *Paragonimus* eggs from stools of paragonimiasis patients by AMS III centrifuging technic. *Jpn. J. Med. Sci. Biol.* 6, 207, 1953.
50. Young, K.H. et al. Ethyl acetate as a substitute for diethyl ether in the formalin-ether sedimentation technique. *J. Clin. Microbiol.* 10, 852, 1979.
51. Kuroki, M. et al. High-resolution computed tomography findings of *P. westermani. J. Thorac. Imaging* 20, 210, 2005.
52. Song, H.O. et al. Skin test for paragonimiasis among schoolchildren and villagers in Namback District, Luangprabang province, Lao PDR. *Korean J. Parasitol.* 46, 179, 2008.
53. Sawada, T., Takei, K., and Yoneyama, K. Studies on the immunodiagnosis of paragonimiasis. II. Intradermal tests with fractionated antigens. *J. Infect. Dis.* 114, 315, 1964.
54. Yokogawa, M., and Tsuji, M. Immunological diagnosis as the screening method of paragonimiasis in the endemic area of paragonimiasis. In: *First Regional Symposium on Scientific Knowledge of Tropical Parasites, University of Singapore, 5 to 9 November, 1962*, UNESCO, Singapore, p. 194, 1962.
55. Biguet, J. et al. Contribution of immunoelectrophoretic analysis to the knowledge of worm antigens: Practical effects on their standardization, their purification and diagnosis of helminthiasis by immunoelectrophoresis. *Rev. Immunol. Ther. Antimicrob.* 29, 5, 1965.
56. Katamine, D., Imai, J., and Iwamoto, I. Immunological study on paragonimiasis: Agar-gel precipitin reactions in paragonimiasis. *Trop. Med.* 10, 28, 1968.
57. Tsuji, M., Capron, A., and Yokogawa, M. Immunodiffusion test in the diagnosis of paragonimiasis. In: *Second International Congress of Parasitology (ICOPA II)*, Colloquium on Immunodiagnosis, Washington, D.C., p. 6, 1970.
58. Tsuji, M. On the immunoelectrophoresis for helminthological researches. *Jpn. J. Parasitol.* 23, 335, 1974.
59. Pariyanonda, S. et al. Serodiagnosis of human paragonimiasis caused by *Paragonimus heterotremus. Southeast Asian J. Trop. Med. Public Health* 21, 103, 1990.
60. Quicho, L.P. et al. Humoral immune response of cats to *Paragonimus* infection. *Southeast Asian J. Trop. Med. Public Health* 12, 364, 1981.
61. Waikagul, J. Serodiagnosis of paragonimiasis by enzyme-linked immunosorbent assay and immunoelectrophoresis. *Southeast Asian J. Trop. Med. Public Health* 20, 243, 1989.
62. Imai, J. Evaluation of ELISA for the diagnosis of *paragonimiasis westermani Trans. R. Soc. Trop. Med. Hyg.* 81, 3, 1987.
63. Zhang, Z. et al. Diagnosis of active *Paragonimus westermani* infections with monoclonal antibody-based antigen detection assay. *Am. J. Trop. Med. Hyg.* 49, 329, 1993.
64. Itoh, M., and Sato, S. Multi-dot enzyme-linked immunosorbent assay for serodiagnosis of trematodiasis. *Southeast Asian J. Trop. Med. Public Health* 21, 471, 1990.
65. Indrawati, I. et al. Studies on immunodiagnosis of human paragonimiasis and specific antigen of *Paragonimus heterotremus. Int. J. Parasitol.* 21, 395, 1991.
66. Narain, K., Devi, K.R., and Mahanta, J. Development of enzyme-linked immunosorbent assay for serodiagnosis of human paragonimiasis. *Indian J. Med. Res.* 121, 739, 2005.
67. Nakamura-Uchiyama, F., Onah, D.N., and Nawa, Y. Clinical features of paragonimiasis cases recently found in Japan: Parasite-specific immunoglobulin M and G antibody classes. *Clin. Infect. Dis.* 32, e171, 2001.
68. Ikeda, T. et al. Parasite-specific IgE and IgG levels in the serum and pleural effusion of *Paragonimiasis westermani* patients. *Am. J. Trop. Med. Hyg.* 47, 104, 1992.
69. Sugiyama, H. et al. Characterization and localization of *Paragonimus westermani* antigen stimulating antibody formation in both the infected cat and rat. *J. Parasitol.* 73, 363, 1987.
70. Maleewong, W. et al. Antigenic components of *Paragonimus heterotremus* recognized by infected human serum. *Parasite Immunol.* 13, 89, 1991.
71. Maleewong, W. et al. Analysis of antibody levels before and after praziquantel treatment in human paragonimiasis heterotremus. *Asian Pac. J. Allergy Immunol.* 10, 69, 1992.
72. Dekumyoy, P., Waikagul, J., and Eom, K.S. Human lung fluke *Paragonimus heterotremus*: Differential diagnosis between *Paragonimus heterotremus* and *Paragonimus westermani* infections by EITB. *Trop. Med. Int. Health* 3, 52, 1998.
73. Kong, Y. et al. Immunoglobulin G (IgG) subclass and IgE responses in human paragonimiasis caused by three different species. *Clin. Diagn. Lab. Immunol.* 5, 474, 1998.
74. Gan, X.X. et al. Development of rapid diagnostic kit (Dot Immunogold Filtration Assay) for detection of antibodies against *Paragonimus westermani. Chin. J. Zoo.* 21, 988, 2005.
75. Qian, B.Z., and Sugiyama, H. Development of an immunological diagnostic kit for paragonimiasis and its use for an epidemiological survey of zoonotic paragonimiasis in Mainland China, 1. Multi-antigen dot immunogold filtration assay (Multi-DIGFA) for serodiagnosis of paragonimiasis. Final report of the JHSF project, The Japan Health Sciences Foundation, Tokyo, 2007.
76. Singh, T.S. et al. Morphological and molecular characterizations of *Paragonimus heterotremus*, the causative agent of human paragonimiasis in India. *Southeast Asian J. Trop. Med. Public Health* 38(Suppl. 1), 82, 2007.
77. Chang, Z.S. et al. Gene sequencing for identification of *Paragonimus* eggs from a human case. *Chin. J. Parasitol. Parasitic Dis.* 18, 213, 2000.
78. Le, T.H. et al. *Paragonimus heterotremus* Chen and Hsia, (1964), in Vietnam: A molecular identification and relationships of isolates from different hosts and geographical origins. *Acta Trop.* 98, 25, 2006.
79. Intapan, P.M. et al. Detection of *Paragonimus heterotremus* eggs in experimentally infected cats by a polymerase chain reaction-based method. *J. Parasitol.* 91, 195, 2005.
80. Nkouawa, A. et al. Paragonimiasis in Cameroon: Molecular identification, serodiagnosis and clinical manifestations. *Trans. R. Soc. Trop. Med. Hyg.* 103, 255, 2009.
81. Agatsuma, T. et al. Mitochondrial DNA differentiation of Japanese diploid and triploid *Paragonimus westermani. J. Helminthol.* 68, 7, 1994.
82. Sugiyama, H. et al. Molecular discrimination between individual metacercariae of *Paragonimus heterotremus* and *P. westermani* occurring in Thailand. *Southeast Asian J. Trop. Med. Public Health* 36(Suppl. 4), 102, 2005.
83. Sugiyama, H. et al. A multiplex PCR for discrimination between *Paragonimus westermani* and *P. miyazakii* at the

metacercarial stage. *Southeast Asian J. Trop. Med. Public Health* 35(Suppl. 1), 327, 2004.
84. Intapan, P.M. et al. Genomic characterization of lung flukes, *Paragonimus heterotremus*, *P. siamensis*, *P. harinasutai*, *P. westermani* and *P. bangkokensis* by RAPD markers. *Vet. Parasitol.* 124, 55, 2004.
85. Maleewong, W. et al. Detection of *Paragonimus heterotremus* in experimentally infected cat feces by antigen capture-ELISA and by DNA hybridization. *J. Parasitol.* 83, 1075, 1997.
86. Sugiyama, H. et al. Polymerase chain reaction (PCR)-based molecular discrimination between *Paragonimus westermani* and *P. miyazakii* at the metacercarial stage. *Mol. Cell. Probes* 16, 231, 2002.
87. Rowan, W.B. The mode of hatching of the egg of *Fasciola hepatica. Exp. Parasitol.* 5, 118, 1956.
88. Vanijanonta, S. et al. Pulmonary paragonimiasis with expectoration of worms: A case report. *Southeast Asian J. Trop. Med. Public Health* 12, 104, 1981.
89. Miyazaki, I., Arellano, R.C., and Grados, O. The first demonstration of the fluke, *Paragonimus* from man in Peru. *Jpn. J. Parasitol.* 21, 168, 1972.
90. Miyazaki, I., and Fontan, R. Mature *Paragonimus heterotremus* found from a man in Laos. *Jpn. J. Parasitol.* 19, 109, 1970.
91. Miyazaki, I., and Harinasuta, T. The first case of human paragonimiasis caused by *P. heterotremus* Chen et Hsia, 1964. *Ann. Trop. Med. Parasitol.* 60, 509, 1966.
92. Sugiyama, H. et al. Studies on the geographical distribution of the lung fluke. *Paragonimus westermani* (Kerbert, 1878) in the southern prefectures of the Kinki District, Japan: Observations on the incidence of encysted larvae of *P. westermani* in *Geothelphusa dehaani* in Higashi-Yoshino area, Nara Prefecture. *J. Vet. Med. Sci.* 45, 227, 1983.
93. Maejima, J., Kamo, H., and Hatsushika, R. Studies on *Paragonimus miyazakii* Kamo, Nishida, Hatsushika et Tomimura, 1961. 2. Second intermediate host and intracrab stage. *Yonago Acta Med.* 15, 35, 1971.
94. Komalamisra, C., Asavisanu, R., and Setasuban, P. Distribution of *Paragonimus heterotremus* metacercariae in fresh water crab, *Tiwaripotamon beusekomae* Bott 1970. *Southeast Asian J. Trop. Med. Public Health* 19, 337, 1988.
95. Rangsiruji, A. et al. A new record of *Paragonimus* other than *P. westermani* in southern Thailand. *Southeast Asian J. Trop. Med. Public Health* 37(Suppl. 3), 57, 2006.
96. Bowles, J., Blair, D., and McManus, D.P. A molecular phylogeny of the human schistosomes. *Mol. Phylogenet. Evol.* 4, 103, 1995.
97. Rim, H.J. Paragonimiasis: Experimental and clinical experience with praziquantel in Korea. *Arzneimittelforschung* 34(9B), 1197, 1984.
98. Nawa, Y., and Doanh, P.N. *Paragonimus bangkokensis* and *P. harinasutai*: Reappraisal of phylogenetic status. *J. Trop. Med. Parasitol.* 32, 87, 2009.