

Molecular identification and epidemiological characterization of cryptosporidiosis and fasciolosis in central Vietnam

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URL	http://hdl.handle.net/10097/59579

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学位の種類 博士（農学）

学位記番号 農第781号

学位授与年月日 平成25年9月12日

学位授与の要件 学位規則第4条第2項

最終学歴

論文題目 Molecular identification and epidemiological characterization of
cryptosporidiosis and fasciolosis in central Vietnam

(ベトナム中部におけるクリプトスポリジウム症および肝蛭症の分子疫学的
研究)

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論 文 內 容 要 旨

Title of the thesis:

Molecular identification and epidemiological characterization of Cryptosporidiosis and Fasciolosis in central Vietnam

Cryptosporidiosis is an important cause of diarrhea in humans and livestock, mainly in young and neonatal individuals. The effect of infection varies greatly, including mild to severe diarrhea, weight loss and dehydration, sometimes leading to mortality. Fasciolosis is an important parasitic disease of livestock, particularly of cattle, causing significant economic losses. Fasciolosis also occurs in humans, resulting in acute and chronic diseases of liver. Both cryptosporidiosis and fasciolosis cause negative social and economic impacts, especially in developing countries, including Vietnam.

The aim of this thesis is to obtain a greater understanding of the epidemiology of cryptosporidiosis and fasciolosis in livestock in Vietnam by: (i) estimating the prevalence of cryptosporidiosis and fasciolosis in common domestic animals in central Vietnam (ii) identifying which *Cryptosporidium* species/genotypes and which *Fasciola* spp. harboring in domestic animals in central Vietnam (iii) assessing the zoonotic importance of *Cryptosporidium* spp. and *Fasciola* spp., evaluating the current situation of cryptosporidiosis and fasciolosis in domestic animals in central Vietnam.

Chapter 1

Prevalence and molecular characterization of *Cryptosporidium* in native beef calves in central Vietnam

In this chapter, a cross-sectional survey was conducted, the aims of which were to investigate the prevalence of *Cryptosporidium* and to characterize the genotype distribution of *Cryptosporidium* isolates in native beef calves 2-6 months old in central Vietnam. The overall prevalence on the sample and herd levels were 18.9% (44/232) and 50% (20/40), respectively. Genotyping based on PCR and sequence analysis of the 18S rRNA gene revealed occurrence of the two non-zoonotic species *Cryptosporidium ryanae* and *C. bovis*, with the former as a dominant species in the animals. The absence of the zoonotic species *C. parvum* in calves examined suggests that the native beef calves 2-6

months old in the study area are unlikely to contribute to human cryptosporidiosis transmission.

Chapter 2

Prevalence, risk factors and molecular characterization of *Cryptosporidium* in pigs in central Vietnam

Part 1: Prevalence and risk factors associated with *Cryptosporidium* oocyst shedding in pigs in central Vietnam

In **chapter 2**, prevalence, risk factors and molecular characterization of *Cryptosporidium* in pigs in central Vietnam were studied. In **part 1**, a cross-sectional survey was conducted. A total of 740 single fecal samples collected from diarrheic and non-diarrheic pigs on 89 farms were screened by the modified Ziehl-Neelsen staining method. Prevalence at the animal and the farm levels were 18.1% (134/740) and 71.9% (64/89), respectively. Risk factors for the infection were identified using univariate and multivariate logistic regression analysis. The results revealed that age, sanitary condition and topography were significantly associated with oocyst shedding ($P < 0.05$). There was an association between the occurrence of diarrhea and the level of *Cryptosporidium* oocyst excretion within infected pigs. This is the first epidemiological investigation of prevalence and risk factors of *Cryptosporidium* in pigs in Vietnam.

Part 2: Molecular characterization of *Cryptosporidium* in pigs in central Vietnam

In **part 2**, the genotype distribution of *Cryptosporidium* isolates in pigs in Vietnam was characterized. A total of 193 pig fecal samples were screened for the presence of *Cryptosporidium* oocysts using the modified Ziehl-Neelsen staining method, and 28 (overall prevalence 14.5%) were identified as positive by microscopic observation. Genetic identification based on the 18S ribosomal RNA and 70 kDa heat shock protein genes revealed that pigs in Vietnam are infected with two species/genotypes (*Cryptosporidium suis* and *Cryptosporidium* pig genotype II). The presence of these host-adapted species/genotypes suggests that pigs may not pose a significant public health risk in this area.

Chapter 3

Prevalence and molecular characterization of *Cryptosporidium* in ostriches (*Struthio camelus*) on a farm in central Vietnam

In **chapter 3**, prevalence and molecular characterization of *Cryptosporidium* in ostriches on a farm in Khanh Hoa province, central Vietnam were determined. A total of 464 ostrich fecal samples were examined *Cryptosporidium* oocysts, and 110 (overall prevalence 23.7%) were identified as positive by microscopy. Prevalence of *Cryptosporidium* in animals of <45 days, 45-60 days, 61-90 days, 91 days-12 months and >12 months was 23.5% (16/68), 33.3% (22/66), 35.2% (68/193), 0 and 5.8% (4/69), respectively ($p < 0.05$). The majority of positive samples scored as the 3+ level of intensity of infection were from 61-90 days ostriches. Molecular analysis in the 18S ribosomal RNA, 70 kDa heat shock protein and actin genes demonstrated the presence of only *Cryptosporidium* avian genotype II in ostriches in central Vietnam.

Chapter 4

Prevalence and molecular identification of Fasciolosis in cattle in central Vietnam

Part 1: Molecular identification of *Fasciola* spp. (Digenea: Platyhelminthes) in cattle from Vietnam

In **chapter 4**, prevalence of *Fasciola* in cattle and of its intermediate host *Lymnaea* snails in central Vietnam was studied, and the genetic identification of *Fasciola* was described. In **part 1**, molecular characterization of *Fasciola* in cattle from central Vietnam was described. *Fasciola* spp. were collected from naturally infected cattle in Khanh Hoa province, Vietnam for morphological and genetic investigations. Microscopic examination detected no sperm cells in the seminal vesicles, suggesting a parthenogenetic reproduction of the flukes. Analyses of sequences from the first and second internal transcribed spacers (ITS1 and ITS2) of the ribosomal RNA revealed that 13 out of 16 isolates were of *Fasciola gigantica* type, whereas three isolates presented a hybrid sequence from *F. gigantica* and *Fasciola hepatica*. Interestingly, all the mitochondrial sequences (partial COI and NDI) were of *F. gigantica* type, suggesting that the maternal lineage of the hybrid form is from *F. gigantica*. No intra-sequence variation was detected. This result indicated that the genotypic variation also occurred in *Fasciola* population originated from central Vietnam.

Part 2: Prevalence of *Fasciola* in cattle and of its intermediate host *Lymnaea* snails in central Vietnam

In **part 2**, a cross-sectional study were conducted to investigate the prevalence of natural *Fasciola* infections in both the definitive hosts (cattle) and the intermediate hosts (*Lymnaea* snails) in central Vietnam. A total of 1075 fecal samples, randomly collected from cattle in Binh Dinh, Khanh Hoa and Phu Yen provinces, were examined for *Fasciola* eggs by a sedimentation method. The overall prevalence of *Fasciola* was 45.3%. A subset of the animals (235) were also screened for antibodies against *Fasciola* by an Enzyme-Linked Immuno-sorbent Assay. Overall, 46.3% of these animals were shedding *Fasciola* eggs while 87.2% were *Fasciola* seropositive. A lower prevalence of *Fasciola* was observed in calves ≤ 2 years of age (37.6%) compared to that in cattle > 2 years of age (53.7%) ($p < 0.05$). The prevalence in the rainy season (50.8%) was significantly different to that in the dry season (38.1%) ($p < 0.05$). Of the 3,269 *Lymnaea viridis* and 1,128 *Lymnaea swinhoei* examined, 31 (0.95%) and 7 (0.62%), respectively, were found to be infected with *Fasciola*. This appears to be the first epidemiological survey of the prevalence of *Fasciola* in cattle and snails in central Vietnam, where human fasciolosis is hyperendemic in recent years.

The results of present thesis indicated that *Cryptosporidium* and *Fasciola* are highly prevalent in livestock in central Vietnam. For both *Cryptosporidium* and *Fasciola*, cattle is important reservoir for human and animal transmission. For *Cryptosporidium*, pigs and ostriches to a lesser extent are the reservoirs of some host-adapted species/genotypes. Occurrence of bovine, pig and avian specific *Cryptosporidium* species/genotypes in animals examined might implicate that the public health significance of these parasites in livestock in central Vietnam may be minimal. However, the role of other animal species, especially wildlife for *Cryptosporidium* and *Fasciola* are unclear and requires further studies. Control of *Cryptosporidium* and *Fasciola* might achieve through effective chemotherapy combined with good management strategy and good hygienic measures.

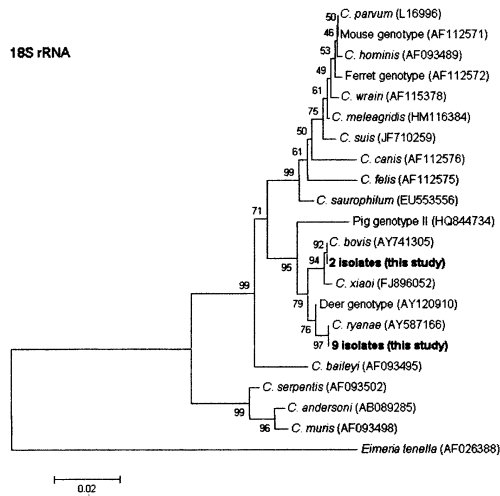


Fig. 1. Phylogenetic relationship of *Cryptosporidium* isolates in Vietnamese cattle using partial 18S rRNA sequences. Accession numbers are shown in parentheses

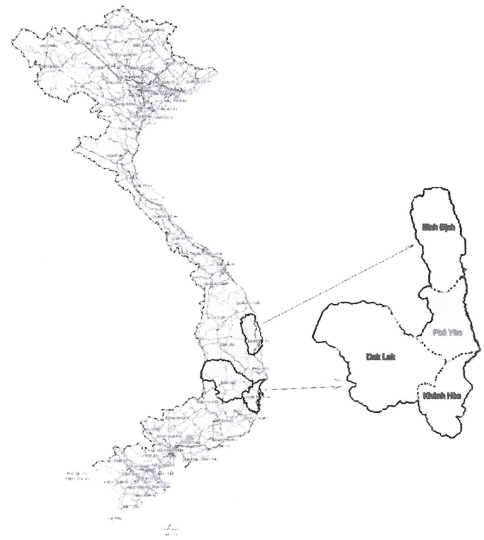


Fig. 2. Map of sampling locations in central Vietnam. The provinces of BinhDinh, KhanhHoa are coastal-delta zones and DaLac is mountainous zone.

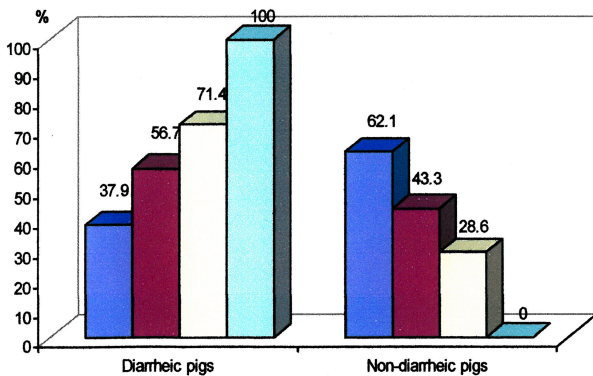


Fig. 3. Association between the presence of diarrhea and the level of oocyst excretion within infected pigs

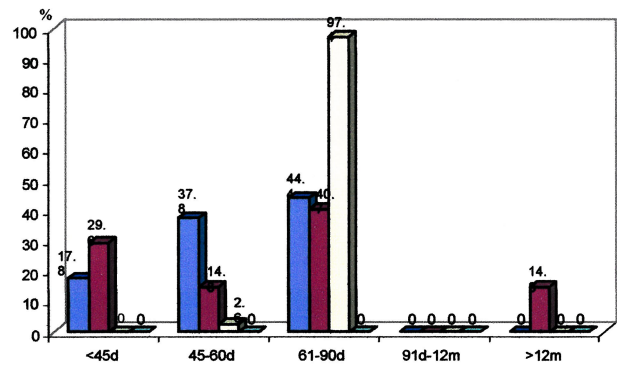


Fig. 4. Relationship between different age categories and intensity of *Cryptosporidium* infection in infected ostriches (d=days, m=months)

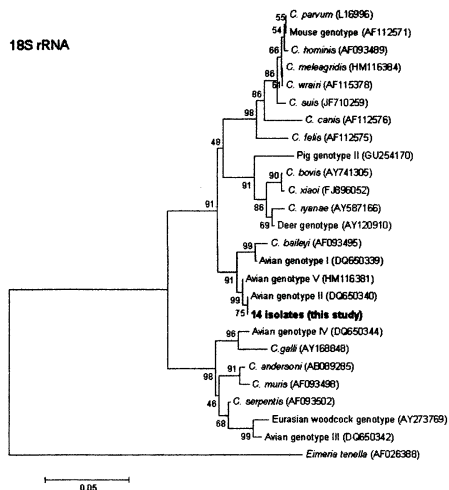


Fig. 5. Phylogenetic relationships of ostrich *Cryptosporidium* isolates using partial 18S rRNA gene sequences. Accession numbers are shown in parentheses.

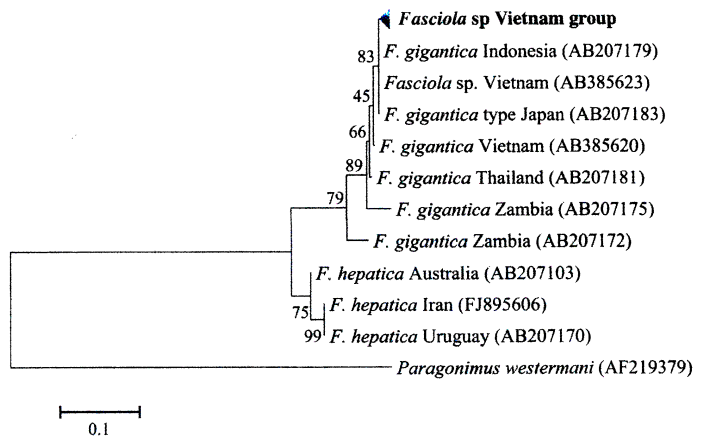


Fig. 6. Phylogenetic relationship of *Fasciola* parasites based on sequences of COI. *Paragonimus westermani* (AF219379) was used as an outgroup.

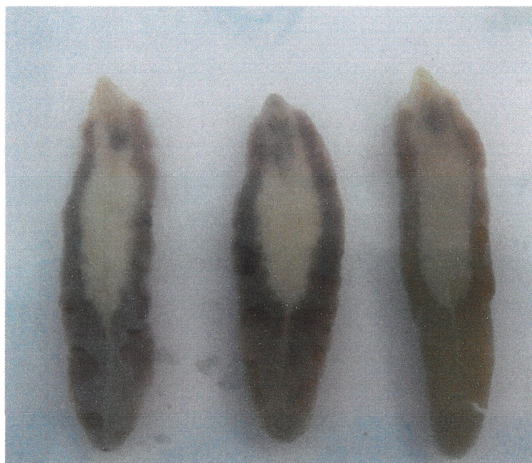


Fig. 7. *Fasciola* adult worms collected in abattoirs in Khanh Hoa province

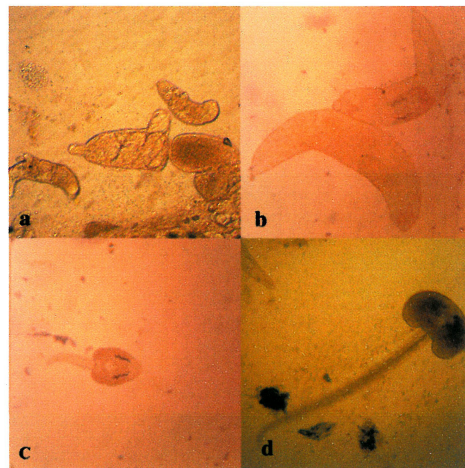


Fig. 8. *Fasciola* larval forms found in *Lymnaea* snails. All x400. (a) Sporocysts. (b) Redia. (c) Young cercaria. (d) Adult cercaria.

論文審査結果要旨

Nguyen Thi Sam 氏は、JSPS の RONPAKU 研究員として、4 年半にわたって、寄生虫の分子疫学研究を行ってきた。勤務先であるベトナム中央獣医学研究所でももにフィールド調査および寄生虫のサンプリングを実施し、毎年、約 100 日間来日して、環境システム生物学分野において遺伝子解析を実施し、これらの成果を学術雑誌に 6 論文として報告してきた。本学位論文は、これらの報告をまとめたものである。

ベトナムでは、近年、経済規模が急速に拡大し、家畜生産量が増えつつある。家畜頭羽数の拡大に畜産における衛生環境の整備は伴わず、家畜感染症および人獣共通感染症が大きな問題となっている。本研究では、人獣共通に感染する原虫および蠕虫として、クリプトスポリジウムおよび肝蛭に注目して、ベトナム中央部でのこれらの寄生虫の家畜への感染状況を調査し、遺伝子解析により感染種の同定を行ったものである。

クリプトスポリジウム症は、ヒトおよび家畜に下痢を引き起こす原虫であるクリプトスポリジウムによる疾病で、飲料水を介した大規模な集団感染が先進国でも見られる重要な伝染病である。肝蛭は、ウシなどの肝臓に寄生する蠕虫であり、糞中に排泄された虫卵はヒメモノアラガイなどの貝を中間宿主として成長し、水草などを介してヒトや動物に感染する感染症である。ヒトでは肝機能障害を引き起こし、重要な人獣共通感染症である。

本研究においては、従来から行われていた検鏡法を用いて、野外サンプルからの原虫オシストおよび虫卵の検出を行って、農場および家畜の汚染状況を解析し、分離した寄生虫の DNA を用いた分子生物学的解析を行って、分離株の種同定を詳細に実施した。その結果、ウシにおけるクリプトスポリジウム原虫の感染率は 18.9%であり、その同定された種は *Cryptosporidium ryanae* が主であり、また *C. bovis* も認められた。ブタでは感染率は 14.5% となり、*C. suis* および *Cryptosporidium pig genotype II* が確認された。また近年に飼養が始まったダチョウでは、感染率は 23.7%と高い値であったが、分離された遺伝子型は *Cryptosporidium avian genotype II* のみであった。従って、本研究の調査では人獣共通種は検出されず、すなわち本地域では家畜がヒトクリプトスポリジウム症の原因となる危険性は低いことが明らかとなった。肝蛭では、調査したウシの 87.2%が血清学的陽性であり、また 46.3%の個体から虫卵の排出が認められた。宿主の月齢別調査では、仔牛より成牛の虫卵排出率が高く（仔牛：37.6%，成牛：53.7%）、また季節別では、乾期は 38.1%であったが、雨期の感染率は 50.8%と有意に高く、雨期が肝蛭の感染、または虫卵排出と関連することが示唆された。また、遺伝子解析による同定では、アジアで主に分布する *Fasciola gigantica* が主であったが、*F. gigantica* と *F. hepatica* の雑種も分離された。ミトコンドリアゲノムによる母系遺伝の系譜を確認すると、過去に分離された雑種と同様に、その母系起源は *F. gigantica* であった。

本研究では、ベトナム中央で飼養されるウシ、ブタ、ダチョウから分離された原虫や寄生虫について、種や遺伝子型レベルに至る地理的分布と、他地域との遺伝的関連を明らかにした。また両感染症の原因となる寄生虫や原虫の分布解析に加えて、調査地域における家畜の飼養環境を調べ、それらの飼養環境の要素と原虫や寄生虫の感染率との相関を統計的に解析することにより、この調査地域における感染症のリスク要因へも言及する結果を得た。

これらの Nguyen 氏の研究成果は、すべて国際雑誌に採択・掲載されており、本研究でのクリプトスポリジウム症とカンテツ症における疫学調査で得られた知見は、畜産業から公衆衛生、医学分野まで広範囲に有用なものとなることが期待され、博士（農学）の学位を授与するにふさわしいものと判断した。