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APPLICATION OF MULTIPLEX PCR FOR SPECIES DISCRIMINATION USING INDIVIDUAL METACERCARIAE OF *PARAGONIMUS* OCCURRING IN THAILAND

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SHORT TITLE: Molecular discrimination of Thai *Paragonimus* species

ABSTRACT. The number of lung fluke species documented in Thailand totals six. Of them, *P. heterotremus* is the most important, as it affects humans. Although *P. westermani* is found as metacercariae in the same crab species as *P. heterotremus* in Thailand, human infections with *P. westermani* have not been confirmed. In order to accurately discriminate between individual metacercariae of these two species, we established a multiplex PCR method. Through this method, two products each were amplified from the metacercarial DNA samples of *P. heterotremus* (ca. 310 and 520 bp) and *P. westermani* (ca. 140 and 520 bp). In contrast, 520-bp products alone were found to be generated from the DNA samples of *P. siamensis*, *P. bangkokensis* and *P. harinasutai*, three other species of lung flukes known to occur in Thailand. Digestion of these 520-bp products with the restriction enzyme *Scr*FI could unequivocally discriminate species by the number and size of the produced band(s): three bands (ca. 60, 210 and 250 bp) for *P. harinasutai*, two bands (ca. 250 and 270 bp) for *P. bangkokensis*, and an uncut band (520 bp) for

P. siamensis. The established multiplex PCR used in combination with restriction enzyme digestion (PCR-RFLP with *Scr*FI) is effective for discriminating the five different species of the lung flukes occurring in Thailand, even at the metacercarial stage.

INTRODUCTION

The number of lung fluke species documented in Thailand totals six (Blair *et al*, 1999; Srisont *et al*, 1997): *Paragonimus westermani*, *P. siamensis*, *P. heterotremus*, *P. bangkokensis*, *P. macrorchis* and *P. harinasutai*. Of them, *P. heterotremus* is the most important, as it affects humans. Although *P. westermani* occurs as metacercariae in the same crab species as *P. heterotremus* in Thailand, human infections with *P. westermani* have not been confirmed (Blair *et al*, 1998). In order to accurately discriminate between individual metacercariae of these two species, we established a multiplex PCR method (Sugiyama *et al*, 2005). In this study, we further evaluated the usefulness of the previously established multiplex PCR for species-level discrimination among *P. siamensis*, *P. bangkokensis* and *P. harinasutai*, three other species of lung flukes known to occur in Thailand.

MATERIALS AND METHODS

Parasite samples and DNA isolation

The metacercariae of *P. siamensis* (Fig. 1) were harvested from the freshwater crab, *Sayamia germaini*, captured in paddy fields in Prachin Buri Province, Thailand (Srisont *et al*, 1997). The metacercariae of *P. bangkokensis* (Fig. 2) were harvested from the freshwater crab, *Ranguna smalleyi*, captured in a mountain stream in Surat Thani Province, Thailand (Rangsiruji *et al*, in press). The metacercariae of *P. heterotremus*, *P. westermani* (strain Thailand) and *P. harinasutai* (Fig. 3) were harvested from the freshwater crab, *Larnaudia larnaudii*, captured in a mountain stream in Saraburi Province, Thailand (Kawashima *et al*, 1989). DNA samples were prepared from the metacercariae as previously described (Sugiyama *et al*, 2002).

DNA amplification and sequencing

For multiplex PCR amplification (Sugiyama *et al*, 2005), the *P.*

heterotremus-specific forward primer (PhTF1; 5'-TTCCCCAACGTGGCCTTGTGT-3', alignment positions 184 to 204 for the *P. heterotremus* second internal transcribed spacer (ITS2) region of the nuclear ribosomal DNA (rDNA)) and a newly designed *P. westermani*-specific forward primer (PwTF3; 5'-GTCTGCGTTCGATGCTGACCTACG-3', alignment positions 367 to 390 for the *P. westermani* ITS2 region) were used in combination with an interspecies-conserved primer pair, 3S (forward, 5'-GGTACCGGTGGATCACTCGGCTCGTG-3') and A28 (reverse, 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3') (Bowles *et al*, 1995). These primers were all incorporated into a single-tube reaction. The multiplex PCR amplification was performed as previously described (Sugiyama *et al*, 2004) using 0.1 μM of PhTF1 and PwTF3 primers, 0.5 μM of 3S and A28 primers, 2.5 units of the Taq polymerase (Invitrogen, USA) and 10 ng of the DNA template. The resultant PCR products were separated by electrophoresis on 2% (w/v) agarose gels.

The amplified products were extracted from agarose gels and sequenced using the corresponding primers and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) on an automated sequencer (ABI310, Applied Biosystems). The sequence alignment and comparison were completed using the GENETYX-WIN (ver. 7.0, Software Development Co., Japan) program.

Restriction enzyme digestion of the multiplex PCR products (PCR-linked restriction fragment length polymorphism (PCR-RFLP))

The amplified products (4 μl) were also treated with five units of the restriction enzyme *Hin*cII, *Stu*I or *Scr*FI (New England Biolabs, USA) at 37°C for 12 h. The treated samples were then separated by electrophoresis on 3% (w/v) agarose gels.

RESULTS

Based on the established multiplex PCR method (Sugiyama *et al*, 2005), we confirmed that two products each were amplified from the metacercarial DNA samples of *P. heterotremus* (ca. 310 and 520 bp) and *P. westermani* (ca. 140 and 520 bp) (Fig. 4). On the other hand, 520-bp products alone were generated from the metacercarial DNA samples of *P. siamensis*, *P. bangkokensis* and *P. harinasutai* (Fig. 4). Sequence analysis of the amplified products revealed that the aligned ITS2 region was 463 bp in length in each of the latter three species (Fig. 5).

Similarity searches of the GenBank/EMBL/DDBJ nucleotide databases revealed that the *P. siamensis* and *P. harinasutai* ITS2 sequences were identical to the sequences deposited under accession numbers AF159605 and AF159609, respectively. However, there is no sequence data in the GenBank/EMBL/DDBJ for *P. bangkokensis*, therefore, we deposited the ITS2 region sequence under accession number AB248091.

Pairwise comparisons between *P. siamensis* and each of *P. bangkokensis* and *P. harinasutai* revealed 33 (7.1%) or 34 (7.3%) nucleotide differences, respectively. In contrast, only one (0.2%) nucleotide difference was found between *P. bangkokensis* and *P. harinasutai*.

For species discrimination by PCR-RFLP, the restriction enzymes, *Hin*cII, *Stu*I and *Scr*FI, were selected on the basis of the theoretical restriction maps generated from the ITS2 sequences of *P. siamensis*, *P. bangkokensis* and *P. harinasutai* (Fig. 5). Digestion with *Hin*cII discriminated *P. siamensis* by the restriction pattern of two distinctive bands of about 110 and 410 bp in size, while the 520-bp amplification products of the two other species remained uncut (Fig. 6). In contrast, the PCR product of *P. siamensis* remained uncut by *Stu*I, while those of the two other species were cleaved to produce two bands of about 220 and 300 bp. Digestion with *Scr*FI could unequivocally discriminate *P. siamensis*, *P. bangkokensis* and *P. harinasutai* by number and size of the produced band(s): three bands (ca. 60, 210 and 250 bp) for *P. harinasutai*, two bands (ca. 250 and 270 bp) for *P. bangkokensis*, and an uncut band (520 bp) for *P. siamensis* (Fig. 6).

DISCUSSION

The phylogenetic relationships of the *Paragonimus* species occurring in Thailand have been demonstrated using genetic markers in the ITS2 region of the rDNA (Blair *et al*, 1998; Iwagami *et al*, 2000). In these studies, the ITS2 sequences were amplified by PCR with the primer pair, 3S and A28, from DNA samples prepared from adult worms. Using this primer pair with two other species-specific primers to the previously established multiplex PCR method (Sugiyama *et al*, 2005), we demonstrated that the 520-bp ITS2 sequences alone were generated from the individual metacercariae of *P. siamensis*, *P. bangkokensis* and *P. harinasutai*. Through pairwise comparisons of the sequences of the amplified products, these species were unequivocally discriminated from one another. We then utilized nucleotide differences to select the restriction enzymes *Hin*cII, *Stu*I and *Scr*FI for the PCR-RFLP

analyses, which allowed development of a more rapid and labor-saving discrimination method. Of the restriction enzymes examined, we confirmed that *Scr*FI allowed the most efficient discrimination among these species based on the number and size of the produced band(s). It is noteworthy that the analysis with this enzyme could unequivocally discriminate between *P. bangkokensis* and *P. harinasutai*, which have only a single base difference in the ITS2 region.

In this study, we demonstrated that the previously established multiplex PCR method (Sugiyama *et al*., 2005), when used in combination with the restriction enzyme digestion, is effective for discriminating the five different species of lung flukes occurring in Thailand, even at the metacercarial stage. This method may be applicable to *Paragonimus* occurring in other Asian countries like China and India where sets of *Paragonimus* species that have not yet been studied occur. Further collaborative studies, including evaluation of the usefulness of this method, are now in progress in these areas using locally obtained parasite samples.

ACKNOWLEDGEMENTS

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Legends to Figures

Fig 1. Photomicrograph of fresh *P. siamensis* metacercariae. The metacercariae were encysted by a thick wall and had a spherical shape. The wall thickness averaged 94 μm. The diameter of the cyst ranged from 668 to 736 μm with an average of 701 μm. Bar is 150 μm.

Fig 2. Photomicrograph of fresh *P. bangkokensis* metacercariae. The metacercariae were encysted by a wall and had a spherical to suboval shape. The wall thickness averaged 13 μm. The longitudinal and transverse diameters

of the cyst ranged from 379 to 521 μm and 365 to 469 μm, respectively, with average dimensions of 437 x 422 μm. Bar is 150 μm.

Fig 3. Photomicrograph of fresh *P. harinasutai* metacercariae. The metacercariae were encysted by a wall and exhibited a spherical to suboval shape. The wall thickness averaged 14 μm. The longitudinal and transverse diameters of the cyst ranged from 570 to 748 μm and 534 to 724 μm, respectively, with average dimensions of 655 x 634 μm. Bar is 150 μm.

Fig 4. Results of multiplex PCR amplification of the metacercarial DNA samples from *P. heterotremus* (lane 1), *P. westermani* (lane 2), *P. siamensis* (lane 3), *P. bangkokensis* (lane 4) and *P. harinasutai* (lane 5). Two PCR fragments were amplified from the metacercarial DNA samples of *P. heterotremus* (ca. 310 and 520 bp) and *P. westermani* (ca. 140 and 520 bp). A single 520-bp fragment was produced for *P. siamensis*, *P. bangkokensis* and *P. harinasutai*. A 100-bp DNA ladder was used to estimate the sizes of the bands (lane M).

Fig 5. Aligned sequences of the ITS2 region from *P. siamensis* (Ps), *P. bangkokensis* (Pb) and *P. harinasutai* (Ph) metacercariae. A dot in the *P. bangkokensis* and *P. harinasutai* sequences indicates identity with the *P. siamensis* sequence. The recognition sites of the *Hin*cII (GTT/GAC), *Stu*I (AGG/CCT) and *Scr*FI (CC/CGG and CC/GGG) restriction enzymes are enclosed in boxes. The numbers refer to the lengths of the nucleotide sequences.

Fig 6. Results of RFLP analysis of the ITS2 products amplified from *P. siamensis* (lanes 1, 4 and 7), *P. bangkokensis* (lanes 2, 5, and 8) and *P. harinasutai* (lanes 3, 6, and 9) metacercarial DNA samples. The PCR product of *P. siamensis* (ca. 520 bp) was cleaved with *Hin*cII, producing two bands (ca. 110 and 410 bp, lane 1). The PCR products of the two other species remained uncut (lanes 2 and 3). In contrast, the PCR product of *P. siamensis* remained uncut with *Stu*I (lane 4), while those of the two other species were cleaved to produce two bands (ca. 220 and 300 bp, lanes 5 and 6). Digestion with *Scr*FI discriminated *P. harinasutai* based on the restriction pattern of three distinctive bands (ca. 60, 210 and 250 bp, lane 9). The PCR products of *P. bangkokensis* were cleaved with *Scr*FI to produce two distinctive bands (ca. 250 and 270 bp, lane 8), while those of *P. siamensis* remained uncut (lane 7). Both 25-bp and 100-bp DNA ladders were used to estimate the sizes of the bands (lanes M' and M, respectively).

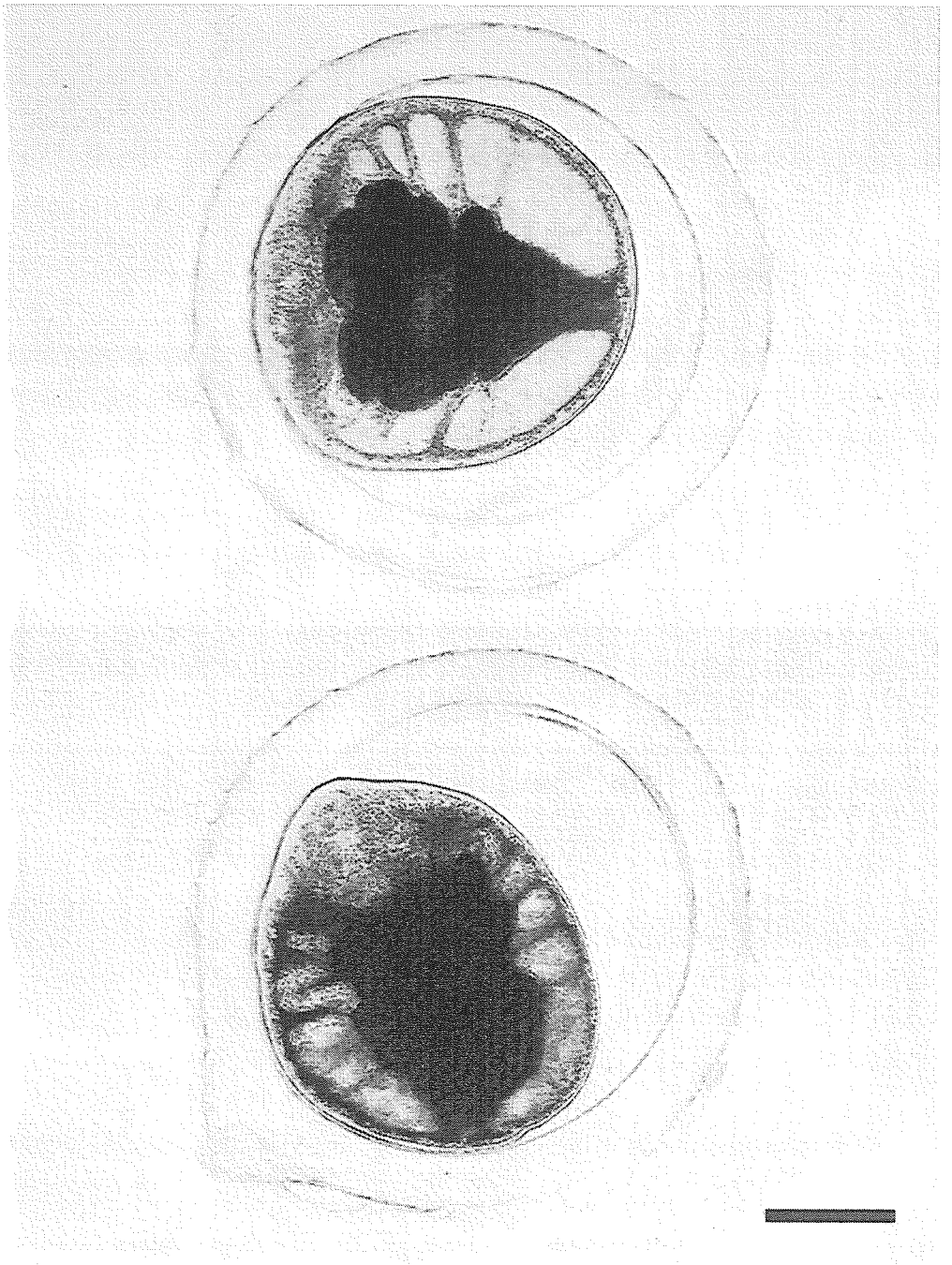

Fig. 1

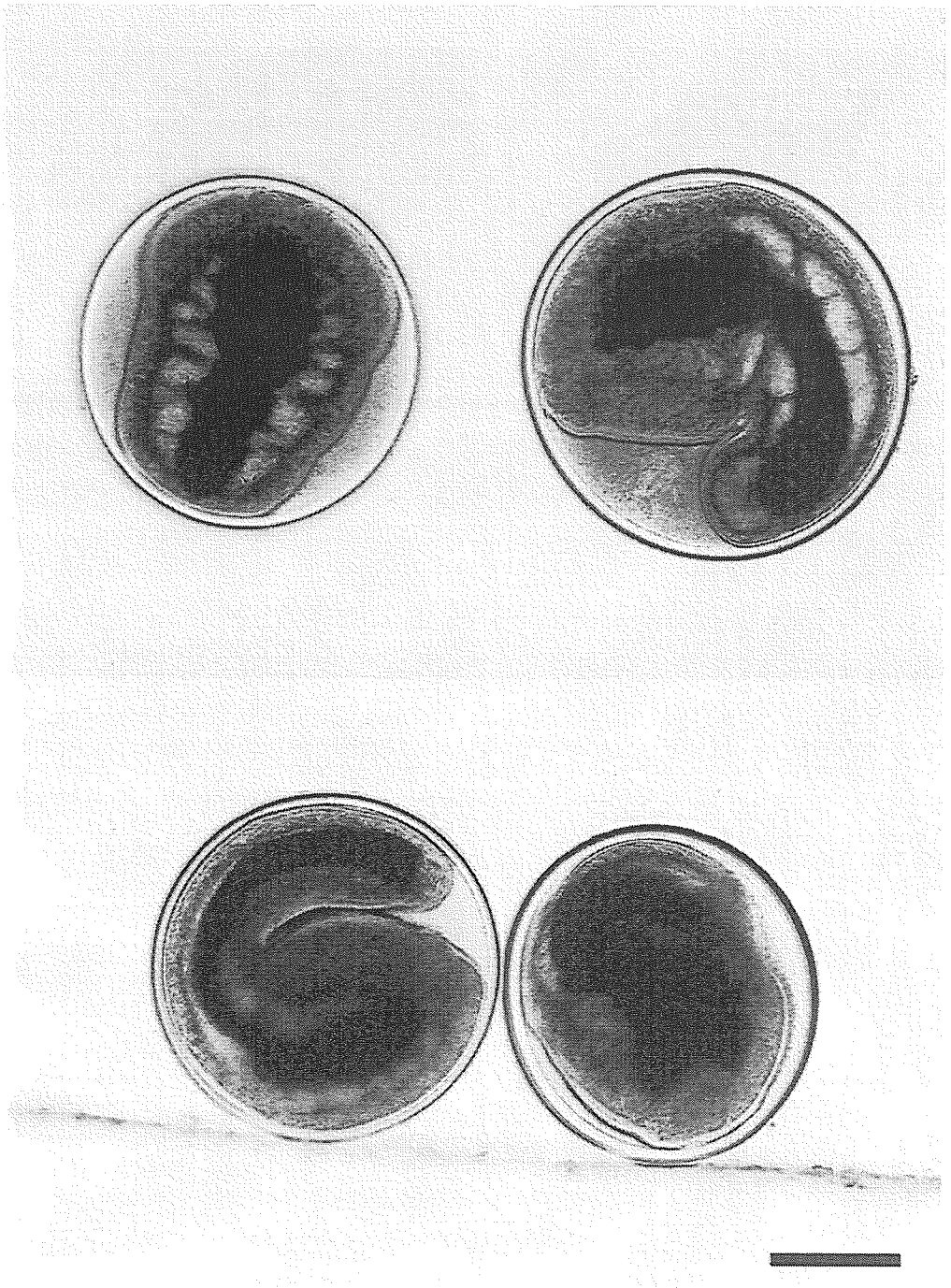

Fig. 2

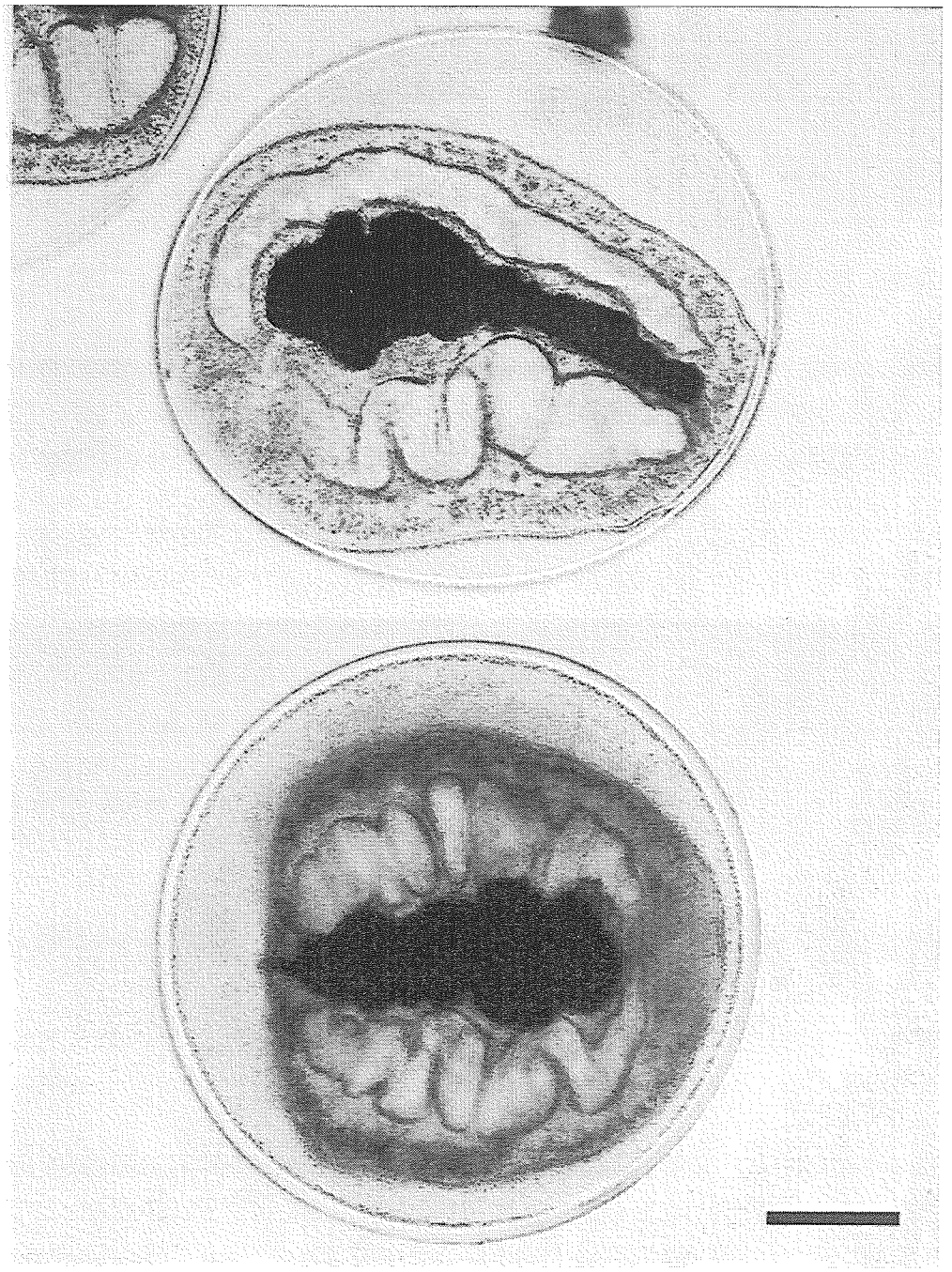

Fig. 3

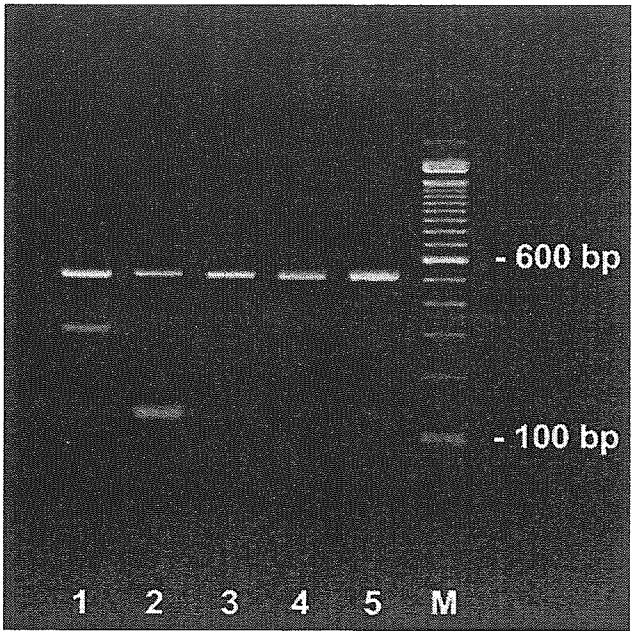

Fig. 4

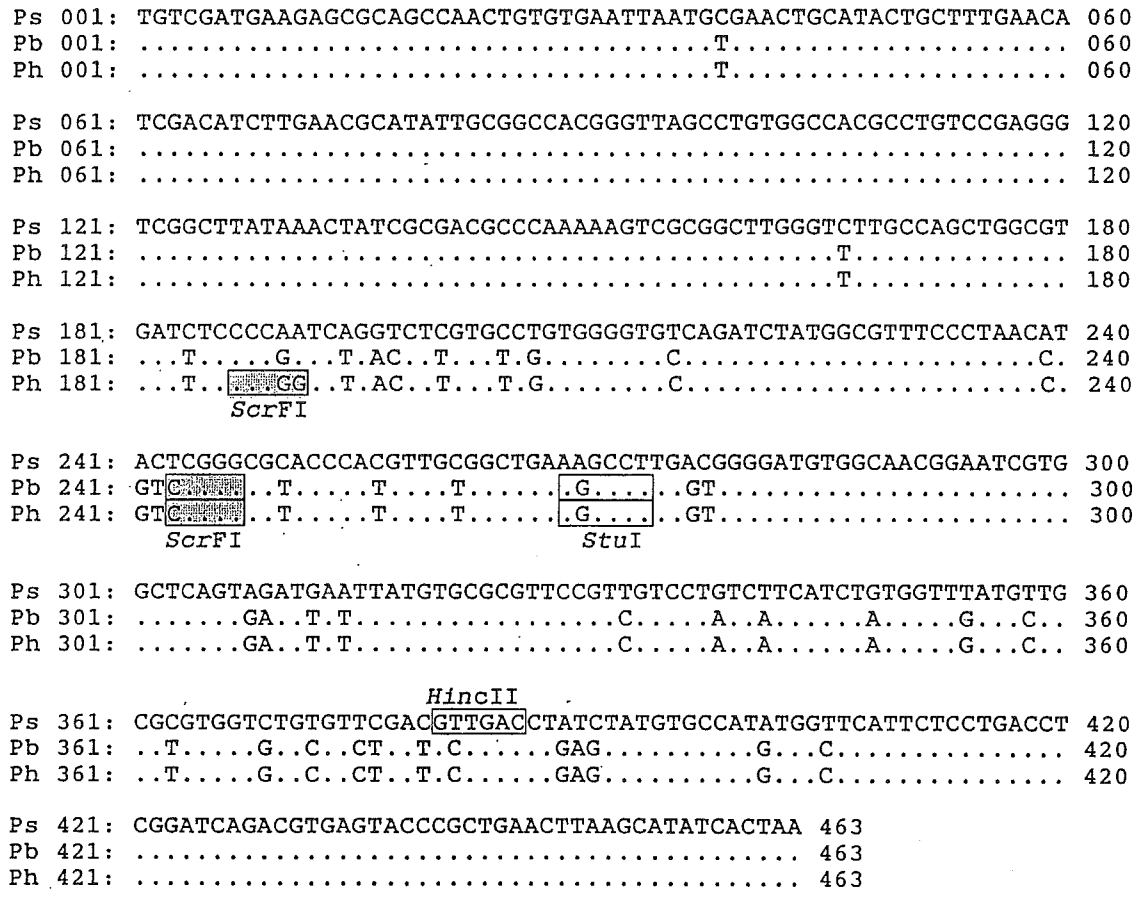
Ps 001: TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACA 060
Pb 001: .....T..... 060
Ph 001: .....T..... 060
Ps 061: TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG 120
Pb 061: 120
Ph 061: 120
Ps 121: TCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTCTTGCCAGCTGGCGT 180
Pb 121: .....T..... 180
Ph 121: .....T..... 180
Ps 181: GATCTCCCCAATCAGGTCTCGTGCCTGTGGGGTGTCAGATCTATGGCGTTTCCCTAACAT 240
Pb 181: ...T.....G...T.AC..T...T.G.....C.....C. 240
Ph 181: ...T..GG..T.AC..T...T.G.....C.....C. 240
ScrFI
Ps 241: ACTCGGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTG 300
Pb 241: GTC..T.....T....T.....G....GT..... 300
Ph 241: GTC..T.....T....T.....G....GT..... 300
ScrFI StuI
Ps 301: GCTCAGTAGATGAATTATGTGCGCGTTCCGTTGTCCTGTCTTCATCTGTGGTTTATGTTG 360
Pb 301: .....GA..T.T.....C.....A..A.....A.....G...C.. 360
Ph 301: .....GA..T.T.....C.....A..A.....A.....G...C.. 360
HincII
Ps 361: CGCGTGGTCTGTGTTCGACGTTGACCTATCTATGTGCCATATGGTTCATTCTCCTGACCT 420
Pb 361: ..T.....G..C..CT..T.C.....GAG.....G...C..... 420
Ph 361: ..T.....G..C..CT..T.C.....GAG.....G...C..... 420
Ps 421: CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA 463
Pb 421: 463
Ph 421: 463

Fig. 5

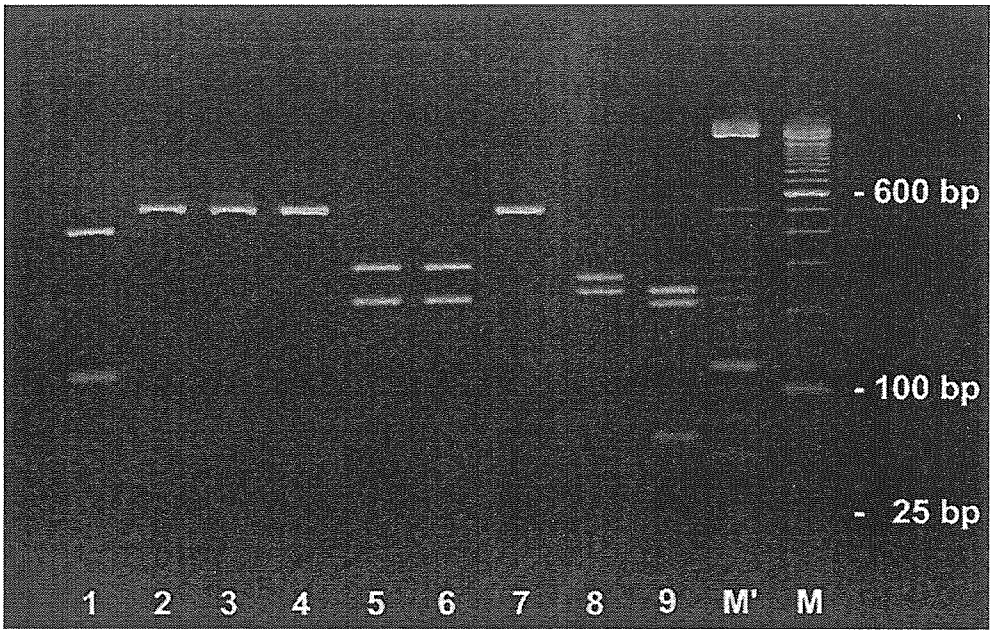

Fig. 6

住血吸虫症を悪化させる宿主側の遺伝要因

Genetic factors associated with hepatic fibrosis of schistosomiasis japonica

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◎疾患遺伝子を考える場合，一般的にとられる方法はその病態生理からもっとも強く病因として疑われる生理活性物質をコードする遺伝子に着目する方法である．感染症では免疫関連遺伝子に着目した解析が広く行われている．現在のようにヒトおよび病原体のゲノムの情報が集積し，感染症の環境要因も明らかになりつつある状況下では遺伝解析を通してゲノムと微生物との相互作用を解明することは十分可能であり，このような解析の結果これまでみえなかったいろいろな相互作用が明らかになることが期待されている．ここでは住血吸虫症重症化にかかわる宿主遺伝要因についての候補遺伝子を用いた解析の結果を報告する．

Key word：日本住血吸虫，肝線維症，HLA，サイトカイン，相乗効果

感染症の外的なストレスは，普通単一の病原微生物でありこれに対する反応パターンが，いわゆる臨床的な感染症として現れるが，この反応パターンには個体差が存在し，まったく症状のない不顕性感染から致死性の重篤な感染症に至る幅広いスペクトラムが観察されている．このような現象が観られるのは反応性を規定する因子が複数存在し，それらが複雑に関与していることによると推測されている．これらの因子をひとつひとつ明らかにしていくことにより感染症というヒトの反応性を理解することが可能となる．

ある疾患遺伝子を考える場合，一般的にとられる方法はその病態生理からもっとも強く病因として疑われる生理活性物質をコードする遺伝子座に着目する方法である．たとえば，神経疾患ではニューロンの機能と関係する物質などがあげられるであろう．感染症では免疫関連遺伝子に着目した解析が広く行われた．そのうちもっとも盛んに行われたのはHLA遺伝子領域である．最近では免疫学の進歩と相まってサイトカインや接着分子などをコードする遺伝子領域の解析も進んでいる．ここでは住血吸虫症にかかわる宿主遺伝要因について候補遺伝子領域からの解析を行った著者らの結果も含めて触れてみたい．

家系調査による遺伝子マッピング

住血吸虫は名前のとおり血液中に寄生する長さ1cmほどの寄生虫で，中間宿主の巻貝で感染型に増殖後湖水や河川，水田などで，ヒトに経皮感染し，約2カ月で成虫となり門脈域に寄生する．多数の卵を腸間膜静脈で産卵し，虫卵を腸管から外界へ旅出する．虫卵の数は1日に数千に及び，しかも無治療では数年間寄生を続けるため慢性あるいは繰り返し感染した患者の約10%で重症の肝線維症あるいは肝硬変を発症し，肝不全や食道静脈破裂などにより死亡する．

経皮感染の際の感染抵抗性については疫学研究から年齢に伴い再感染が軽くなる傾向が観られることから，ある程度の獲得免疫が作用していると考えられている．慢性期の合併症については明らかに個体差の存在することが知られており，10年以上の反復感染歴の後，早い人では30代から重い

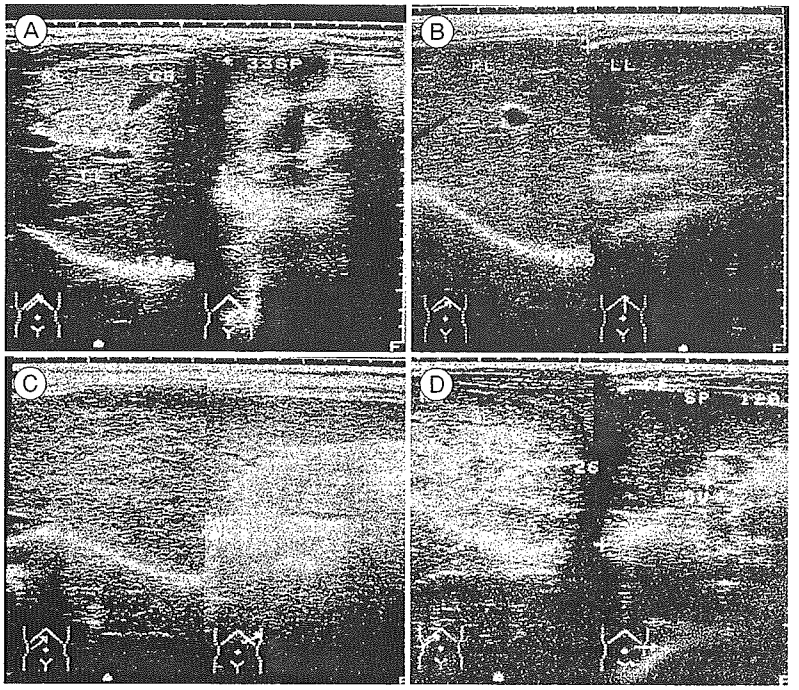

図 1　超音波診断法による住血吸虫性肝線維化症診断像
A：Grade　0，B：Gtade Ⅰ，C：Grade Ⅱ，D：Grade Ⅲ(典型的な亀甲模様を呈する)．

肝硬変となる．住血吸虫症は流行地の環境に根ざした寄生虫疾患で，熱帯地域を中心に3億人ほどの感染者が存在すると推測されている．

住血吸虫症に多かれ少なかれ免疫応答性が関与するのはおそらく間違いないと考えられるが，個々人の抵抗性を定量的に判定し，家系や集団を用いて遺伝要因を解析することはかならずしも容易ではない．Dessein らは1991年ブラジルのマンソン住血吸虫症流行地で，各人の感染抵抗性を虫卵排出数で定量化し，20家系269名の家系調査により共優性の感染性/抵抗性遺伝子座 SM-1 の存在を示唆した[1]．その後，1996年に同じ対象を用いたゲノムワイド解析により SM-1 が 5q31-q33 の CSF1R 付近にマップされることをつきとめた[2]．この近傍には，IL-13，4，5 などの遺伝子座があることから，その本体が注目されているが，まだ明らかになっていない．また，アフリカのスーダンで慢性の住血吸虫性肝線維症の重症群について同様の家系調査を行い 6q22-q23 の IFN-γRI 遺伝子の近傍に肝線維症の感受性遺伝子 SM-2 をマップした[3]．上述したゲノムワイド解析の結果はたしかに驚くべきことで何らかの遺伝子多型が感受性を決定していることを強く示唆するものである．

免疫関連遺伝子との相関

住血吸虫と HLA に関しての論文は著者らとオーストラリアのグループだけであるが，いずれも慢性の肝線維症と HLA-クラスⅡ遺伝子アレルとの強い相関を報告している[4-6]．とくに最近，著者らが見出した HLA-クラスⅡと IL-13B プロモーター遺伝子多型の重症化への相乗効果は驚くべきもので，HLA 多型がたしかに慢性疾患の病型に直結することを示したと考えている．

日本住血吸虫症の流行地として設定したのは中国江西省の山間の村で人口約2,000人のうち30代以上ではほとんどの人が数回の感染を経験していた．1995年にここで10年以上の感染歴のある成人230名を対象に，肝病変の進行度を超音波検査により正常の Grade 0 から肝硬変の Grade 3 までの4段階に診断し(図1)，各人の血液から DNA を抽出し，HLA やサイトカイン遺伝子多型と重症度との相関を解析した．その結果，HLA-DRB1*1101 が進行とともに頻度が下がり逆に HLA-DRB5*0101 と HLA-DPB1＊0301 が重症度で増加する傾向があった[5]．HLA 分子は，T リンパ球へ

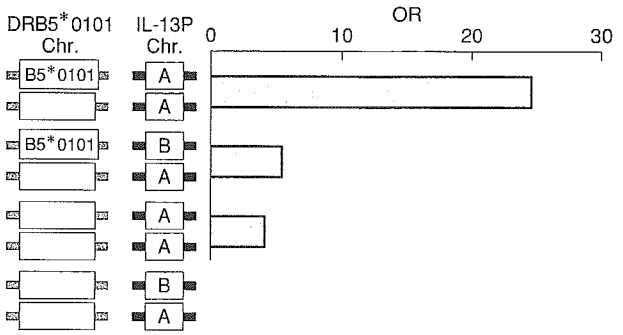

図 2　2つの感受性マーカーである HLA-DRB5*0101 と IL-13P*A/A の相乗効果

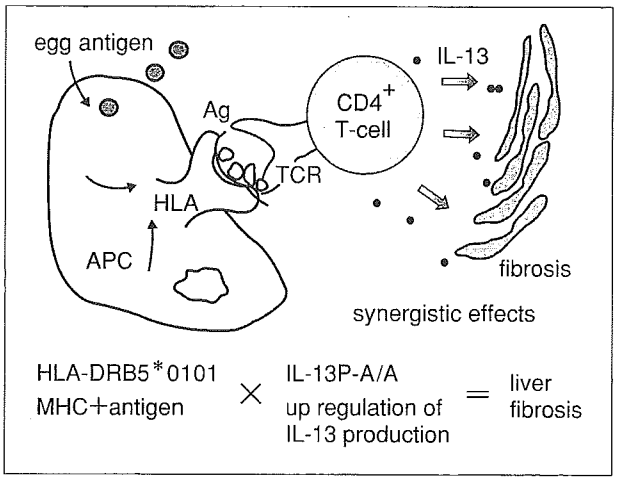

図 3　T ヘルパー 2 モデルによる相乗効果の一元的な説明

の抗原提示分子として働くことから，アレルにより抗原提示能に差が生じ，その結果として肝硬変が現れたと考えられる．一般的にここからは，遺伝子の性格付けから，その機能解析へと展開することになるが，いまのところ，実際にどの抗原分子によってこのような HLA アレル間の反応性の違いが引き起こされるのか明らかではない．

HLA 以外にも TNF，インターフェロン-γや IL-4，IL-13 など Th1，Th2 系のサイトカインの遺伝子領域についても解析したが，唯一，IL-13 のプロモーター領域の SNP ハプロタイプに弱い相関が観られた．以上のように，住血吸虫感染後 HLA-DR アレルおよび IL-13 プロモーター SNP ハプロタイプとの相関が認められたが，これらは，それぞれ第 6，第 5 染色体上に存在するため，これらのマーカーの相互作用について調べたのが図 2 である．感染性マーカーの HLA-DRB5*1010 と IL-13PA/A は同時に存在すると，OR 値が単独の OR 値の和よりはるかに大きく，これらのマーカーが相乗的に作用していることがわかった．それに対して抵抗性マーカーである HLA-DRB1*1101 と IL-13P-B については同時に存在するときの OR 値は各単独 OR 値の和に等しくたがいの相互作用は認められなかった．上記の 2 アレルの解析から図 3 のような肝線維化感受性のメカニズムが推測されている．ただし，最初に紹介したゲノムワイド解析の SM-1(感染抵抗性感受性遺伝子)が IL-13 遺伝子座を含む 5q31-q33 にマップされていたことから，可能性として著者らが見出した肝線維化に対する HLA と IL-13 の相乗効果が実は感染感受性が増した結果，大量の虫体，虫卵に曝露されたために起こったということも考えられる．

おわりに

感染症の遺伝などというのはまだ微生物という概念のない時代の家族集積などを指していたという歴史的な経緯があり，どうも一般に好まれない

サイドメモ

サイトカイン遺伝子多型と疾患感受性

サイトカインは免疫系細胞の情報伝達物質として機能し，免疫応答を調節するネットワークを形成している．サイトカイン遺伝子領域に存在する SNP(一塩基多型)はプロモーター領域であればサイトカイン産生時における発現量の変化を，翻訳領域であれば発現蛋白の変異，サイトカインレセプター遺伝子の翻訳領域における変異はレセプター構造の変異をもたらし，サイトカインネットワークに何らかの異常を生じると考えられる．

このネットワークバランスの乱れによって発症あるいは重症化する疾患として気管支喘息，関節リウマチなどが詳しく調べられており，これらの疾患とさまざまなサイトカイン遺伝子領域多型の相関が報告されている．たとえば，気管支喘息では IL-4 レセプター遺伝子α鎖の細胞外ドメインの 385A→G の変異によって起こる蛋白変異 Q→R の変異，また関節リウマチと TNF-αプロモーター領域の変異の−308A と−857T アレルとの相関がある．

傾向にある．しかし，現在のようにヒトおよび病原体のゲノムの情報が集積し，感染症の環境要因も明らかになりつつある状況下では遺伝解析を通してゲノムと微生物と相互作用を解明することは十分可能であり，このような解析の結果これまで見えなかったいろいろな相互作用が明らかになることが期待される．

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The miniature pig: a unique experimental model for *Schistosoma japonicum* infection

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Abstract

As part of a search for good animal models for human schistosomiasis, two miniature pigs of the CLAWN strain (C-1, C-2) were inoculated percutaneously with 200 *Schistosoma japonicum* cercariae of the Chinese strain, and the subsequent infection was monitored parasitologically, pathologically and serologically. Egg excretion into feces began at 5 weeks post-infection (p.i.) and became pronounced from 8 weeks to 17–20 weeks p.i. The average number of eggs in 1 g feces of each pig at the peak period between 8 and 20 weeks were 288 and 277, respectively. C-1 and C-2 were killed and perfused at 27 and 47 weeks p.i. and adult worm numbers recovered were 35 and 15, respectively. C-2 had at least four pairs of viable mature worms but no detectable fecal eggs for a month before perfusion, suggesting that any produced eggs were not excreted into the feces during this period. Egg deposits associated with inflammatory reactions were observed by histological examination of the liver, spleen, pancreas, mesenteric lymph nodes, lung, and small intestine. This suggests that reduced fecal excretion of eggs into the feces did not correlate to reduced parasite numbers in the chronic phase of schistosomiasis. This is the first report showing the miniature pig to be a potential model for human *S. japonicum* infection.

Keywords: Miniature pig; *Schistosoma japonicum*; Animal model

1. Introduction

Schistosomiasis is one of the major communicable diseases to endanger public health and is of socio-economic importance worldwide. In spite of various efforts to control it, an estimated 200 million people are still infected [1]. In China, the Philippines, and Indonesia, *Schistosoma japonicum* is endemic. Extensive control programs such as snail control, mass chemotherapy, and education have been carried out for over 40 years in these areas and have brought about the control of *S. japonicum* infections in some endemic regions, but large endemic areas still remain in China and the Philippines. In these areas, the presence of reservoir hosts, such as water buffalo and cattle, has made the control more difficult [2,3]. Studies of *S. japonicum* infection in these animals, as well as in man, have been required to overcome this difficulty.

Most of our knowledge about schistosomiasis is drawn from experiments in primates and rodents. Although primates are good hosts for experimental infection [4], the high cost and ethical concerns make them a difficult model for researchers to maintain. The use of rodents has several problems as a model for schistosomiasis. A single worm

Abbreviations: SEA (schistosome soluble egg antigens); SWA (schistosome adult worm antigens); EPG (eggs number per 1 gram feces); MHC (major histocompatibility complex); SLA (swine leukocyte antigen).

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pair in the mouse model affects hepatic blood flow and is said to be equivalent to a burden of approximately 4000 worms in man. The average life span of the schistosomes in humans is longer than that of mice, thereby eliminating their use in studies for long-term effects [5].

Although water buffalo and cattle are the most important reservoir hosts in China, the pig is also known to be another reservoir [2,9]. As an experimental model, it is impractical to use water buffalo and cattle, and, therefore the pig has gained more attention from researchers who are involved in pathology or protective immunity [6–11]. However, the major drawback of standard pigs is the large body mass at 3–4 months old, which reaches more than 100 kg. The schistosome infection needs at least 3 months for researchers to be able to observe its clinical course, and the standard pig's large body size makes handling more difficult compared with other animal models, like rodents. Therefore, smaller pigs, such as the miniature pig, were expected to be ideal. However, Reid and Lichtenberg [12] had already reported that the miniature pig could not serve as an adequate substitute for primates. Following this initial report, miniature pigs have not been used as an animal model. Recently, a new miniature pig strain established in Japan (the CLAWN strain) has become available. Therefore, we evaluated the use of this strain of miniature pigs as a model for human schistosomiasis.

2. Materials and methods

2.1. Experimental animals

The CLAWN strain miniature pigs (Fig. 1) were originally established by Y. Nakanishi during the 1980s and bred in the Japan Farm Clawn Institute (Kagoshima, Japan). Briefly, this pig was developed by crossbreeding two kinds of F1: one is the F1 crossbreed of the Göttingen strain miniature pig and the Ohmini strain miniature pig; and the other is the F1 crossbreed of the Landrace strain pig and the Great Yorkshire strain pig. Generally the weight of the CLAWN miniature pig is approximately 30 kg in the 8-months old and approximately 80 kg in the 24-months old. Two 6-week-old CLAWN strain pigs (C-1 and C-2, 3 kg and 2.5 kg, respectively) were used in this study. The pigs were fed standard nutrient chow based on their body weights, with water ad libitum. The experimental protocol was approved by the Animal Ethical Committee of Nagasaki University.

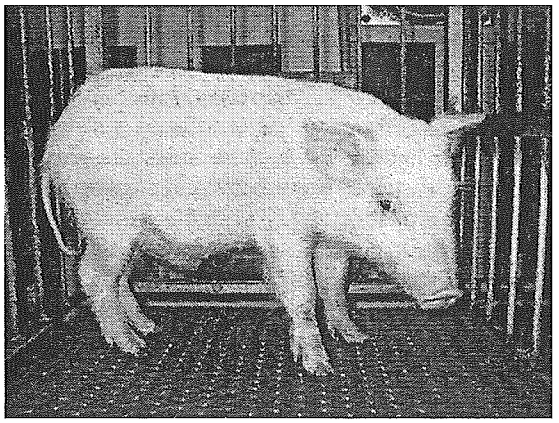

Fig. 1. The CLWAN miniature pig.

2.2. Parasite and parasitological technique

Pigs were percutaneously inoculated with 200 cercariae using a coverslip. The cercariae were shed from infected snails (*Oncomelania hupensis*) with a Chinese strain of *S. japonicum* maintained in the Jiangsu Provincial Institute of Parasitic Diseases Control, Wuxi, Jiangsu Province, People's Republic of China.

Feces were collected every week and the number of eggs excreted into the feces was counted using a method described by Willingham et al. [7]. Briefly, approximately 10 g of feces was homogenized and suspended in 500 ml of 1.2% sodium chloride solution. The feces suspension was poured into a series of three sieves with mesh sizes of 400 μm, 100 μm, and 45 μm, respectively. The residue left on the 45-μm mesh was recovered with 1.2% sodium chloride solution and then centrifuged. One-tenth of the sediment was examined for *S. japonicum* eggs by light microscopy. Eggs per gram of feces (EPG) were calculated from the counts.

2.3. Blood collection

Blood was collected from the auricular vein once a week. Before taking blood, pigs were anesthetized by intramuscular injection with 0.2 mg/kg midazolam (Yamanouchi Pharmaceutical Co. Ltd. Tokyo, Japan) and 40 μg/kg medetomidine (Orion Corp., Espoo, Finland). The ratio of eosinophils number against total white blood cells number were determined on the peripheral blood smear after May-Grünwald Gimsa staining.

2.4. Enzyme-linked immunosorbent assay (ELISA)

An ELISA was performed as described previously [8]. To block the non-specific binding, phosphate buffered saline containing 0.1% blocking agent (Blocking Reagent, 1096176, Roche Diagnostics, Mannheim, Germany) was used.

2.5. Perfusion

S. japonicum adult worms were recovered from the liver and mesenteric veins using a previously described perfusion technique [8]. Pigs were killed by overdose intravenous injection of pentobarbital (30 mg/kg). Heparin sulfate (5000

IU) was also injected intravenously. The thorax and abdomen were cut open by one central longitudinal section from neck to anus. A plastic tube, 1 cm in diameter, was inserted into the descending aorta just above the diaphragm and ligated by silk string just under the renal arteries. Another tube for the flush-out was inserted into the portal vein at the entry to the liver and ligated. Twenty liters of saline containing sodium citrate (15 g/l) was then flushed through the peritoneal vessels. The fluid flushed out of the tube inserted into the portal vein contained the adult worms, and these were captured by the stainless steel mesh. After perfusion, the portal vein was re-examined for residual worms by careful observation.

After recovering the adult worms, the organs were also perfused with periodate–lysine–paraformaldehyde (PLP) solution for fixation. Organs were then excised and immersed in the PLP solution. They were conventionally processed, embedded in paraffin, and sectioned at 3 μm. Sections were stained with hematoxylin–eosin (HE), Masson's trichrome, and the periodic acid Schiff reaction.

A part of liver (left hepatic lobe) was digested in 3% KOH at 37 °C for 24 h. The eggs number in one tenth of the digested fluid was counted to determine the number of eggs per gram of each organ.

3. Results

3.1. S. japonicum infection in miniature pigs

To confirm the establishment of *S. japonicum* infection, fecal egg excretion (EPG) was monitored every week (Fig. 2). Fecal eggs were first detected at five weeks post-infection (p.i.). The kinetics of EPG showed a biphasic pattern. By 8 weeks p.i. in both pigs, EPG increased dramatically to approximately 400. In C-2, EPG increased further to approximately 700 at 10 weeks p.i. The EPG then gradually decreased to approximately 50 at 14–15 weeks p.i. before increasing again. Afterwards, egg excretion persisted at a relatively low level. The eggs excreted at 25 weeks p.i. were still able to hatch in an artificial water pond under light (data not shown). During the infection, the eosinophilia was observed in C-1 at 26 weeks p.i. (9.5%), in C-2 at 13 weeks p.i. (22%).

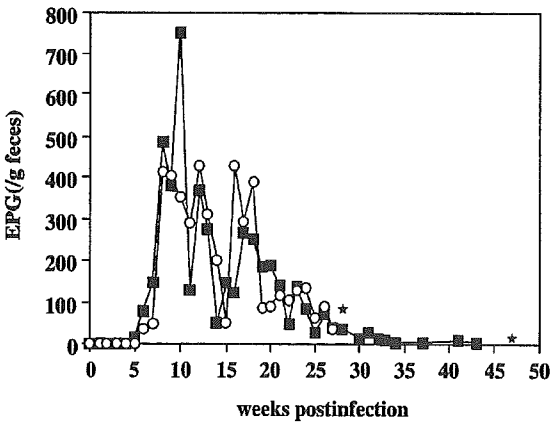

Fig. 2. Fecal egg excretion expressed in eggs per gram of feces of two miniature pigs infected with 200 cercariae of a Chinese strain of *Schistosoma japonicum*. Open circle, data from the C-1 pig. Closed square, data from the C-2 pig. *sacrificed for the perfusion experiment.

C-1 was killed at 27 weeks p.i. to examine the worm recovery. The weight of the pig at the time of killing was 22 kg. The recovered number of worms was 35 (male, 20; female, 13; sex undetermined, 2; 11 paired worms were among these): Thirty-three worms were viable, two worms were dead. The number of eggs in the liver was 361/g liver.

C-2 was killed at 47 weeks p.i. The weight at that time was 42 kg. The adult worms were recovered after portal vein perfusion. The number of worms recovered was 15 (male, 10; female, 5; 4 paired worms were among these). All these worms were alive and all paired female worms had eggs in their uteri. The number of eggs in the liver was 119/g liver.

3.2. Histology

In both pigs, organs (lungs, heart, kidneys, liver, spleen, pancreas, mesenteric lymph nodes, small intestine, large intestine, and brain) were removed and processed for pathological analysis. In C-1 (killed at 27 weeks p.i.), no marked change was macroscopically observed except for moderate enlargement of the mesenteric and portal lymph nodes. Neither apparent fibrosis nor cirrhotic changes were observed in the liver. Histologically, *S. japonicum* eggs were detected in the liver, spleen, pancreas, small intestine, mesenteric lymph nodes, and lungs (Fig. 3a, b, f–h). Deposited eggs were associated with a granulomatous reaction consisting of multinucleated giant cells of foreign body type, epithelioid cells, macrophages, neutrophils, and eosinophils. The granulomatous reaction was surrounded with mild fibrosis and lymphocytic infiltration. The granulomas were localized in and near the portal tract of the liver (Fig. 3a), in the submucosal layer of the small intestine (Fig. 3h), within the peripheral sinus of the mesenteric lymph nodes, and within the vessels of the pancreas and lungs (Fig. 3f, g). Portal–portal bridging fibrosis was occasionally observed in the liver, but lobular disorganization was not apparent.

In C-2 (killed at 47 weeks p.i.), an enlarged spleen and mesenteric lymph nodes, and petechial hemorrhage in the large intestine were observed macroscopically. Histologically, granulomas were found in the liver, pancreas, small intestine, mesenteric lymph nodes, and lungs. The granulomas were relatively small and mainly consisted of epithelioid cells and fibroblasts without being surrounded by eosinophils and lymphocytes (Fig. 3d).