ピラ症をワイル病（黄疸出血性レプトスピラ症）と呼ぶ。ワイル病は日本では血清型Icterohaemorrhagiaeと血清型Copenhageniに起因し、3～14日の潜伏期の後に発病する。この他に中等症、あるいは軽症とされる秋季レプトスピラ症（血清型Autumnalisによる秋疫A症、血清型Hebdomadisによる秋疫B症、血清型Australisによる秋疫C症）がある。

第1期（発熱期）：突然の悪寒を伴う39～40℃に及ぶ発熱に始まり、頭痛、腰痛、全身倦怠感、結膜の充血、腓腹筋痛がおこる。結膜の充血は最も特徴的症状であり（図3）、第2～3病日には顕著となり、第4～5病日には結膜、皮膚に黄疸がみられるようになる。

第2期（発黄期、黄疸期）：黄疸は最高潮に達し、皮膚の点状出血、歯茎や口蓋の口腔内出血、鼻血、吐血、血便、眼球結膜の出血、喀血、血尿などの症状を呈する。

第3期（回復期）：衰弱と激しい貧血がみられ、皮膚は灰緑黄色となる。重症の秋季レプトスピラ症は、ワイル病と臨床症状で区別することができない。秋季レプトスピラ症では、後発症として1～6カ月後に30～40%の頻度で水晶体混濁がみられる。

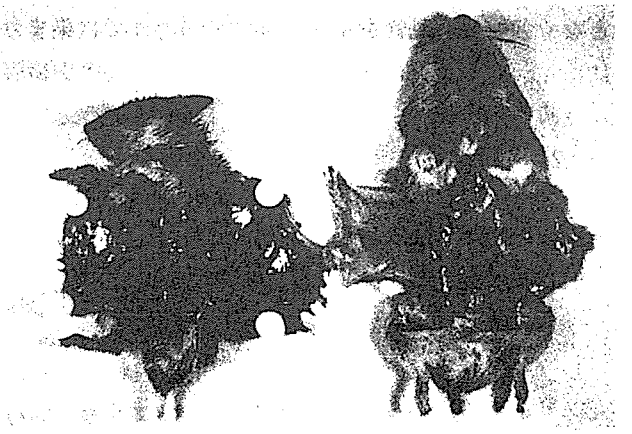
臨床検査所見としては、ヒトでは発病期から蛋白尿、白血球増加、血沈の促進、CRP陽性などの所見がみられる。第1週から2週のはじめにかけて、尿中にレプトスピラの排菌がおこる。重症例では貧血、血小板減少がみられる。黄疸を呈した重症例でも血清中のAST（GOT）、ALT（GPT）、LDHは正常ないし、一過性の上昇にとどまる。

動物のレプトスピラ症

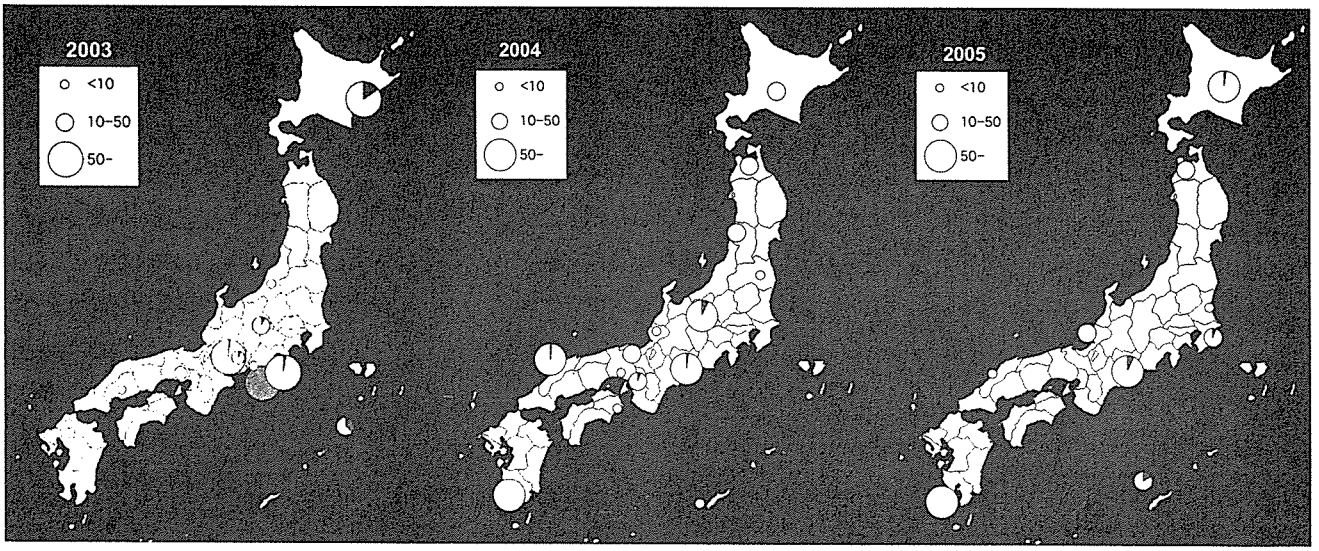
自然界ではレプトスピラは動物の間で循環伝染し、急性感染から回復した動物は尿中にレプトスピラを排菌することがある。また、各種野生動物から家畜、ヒトへ、家畜からヒトへのルートで感染が広がる。一般動物では不顕性感染が圧倒的に多いが、一部の家畜、ペットでは致死的となる。血清型と宿主との間には特異性が見られる。すなわち、イエネズミと血清型IcterohaemorrhagiaeとCopenhageni、ノネズミと血清型Grippotyphosa、イヌと血清型Canicola、ブタと血清型Pomonaのように宿主偏好が認められる（1, 2, 3）。

イヌ型レプトスピラ病は血清型Canicolaによる感染で、保菌率は地域にもよるが6～20%といわれる。イヌでは顕性感染の他に、不顕性感染事例も多く、保菌動物となって数年から生涯に渡り、断続的にレプトスピラを尿中に排菌する（5）。潜伏期はワイル病と同じく4～9日が多い。ワイル病と類似の病形を呈す

【図3】フィリピンで見られたレプトスピラ症患者の結膜の充血、黄疸
第2～3病日に結膜充血が顕著となり、第4～5病日には結膜に黄疸がみられる。早期診断の決め手。（原図：静岡県立大学前教授　柳原　保武）



【図4】実験的レプトスピラ感染ゴールデンハムスターに見られる皮下出血（左）感染動物、（右）正常動物
病原性レプトスピラをゴールデンハムスター腹腔内に接種すると1週間程度で斃死する。皮下や肺に出血と腎臓の著しい変性が観察される。ゴールデンハムスターはげっ歯類にあって例外的にレプトスピラに対する感受性が高い。一方、一般に多くのげっ歯類はレプトスピラに感染しても、病態を呈することはない。同じハムスターでもジャンガリアンハムスターはレプトスピラに抵抗性があった。



【図5】野鼠のレプトスピラ保有率（2003～2005調査）
野鼠を捕獲し、腎臓よりレプトスピラの培養を行った。また分離株の血清型を同定した。
保有率：野鼠45/1101匹（4.1%）北海道、沖縄、長野、愛知（名古屋市内）などで分離
保有野鼠種：*Rattus norvegicus, Apodemus speciosus, A. argenteus, Suncus murinus*
分離血清型：Autumnalis, Hebdomadis, Copenhageni－Icterohaemorrhagiae, Javanica,　未同定血清型

るが、黄疸出血の傾向は弱い。米国ではCanicolaとIcterohaemorrhagiaeに対するワクチンが広く使用されたため、この2血清型のイヌレプトスピラ症はみられず、代わってこのワクチンでカバーされないPomona、Grippotyphosa、Bratislavaが主要な血清型となっている（18）。

ウシでは、日本ではHebdomadis、Autumnalis、欧州ではGrippotyphosaによる血色素尿、米国ではPomonaによる流産が多い。オーストラリアではHardjoによる感染が多い。ブタでは米国ではPomonaによる流産が多く、数か月から1年以上保菌状態となる。ウマは発症しても軽症であることが多いが、流産や重症では合併症として月盲が知られている（19）。この他にキツネ、スカンク、オポッサム、マングース、イノシシ、さらにイエネズミをはじめとする各種げっ歯類は不顕性感染し、保菌動物となる（4, 15）。げっ歯類にあって最もポピュラーなペットであるゴールデンハムスターにレプトスピラを接種すると急性感染をおこし、1週間程度で斃死する。死亡したハムスターでは、血尿、腎臓の著しい傷害、皮下出血などが見られる（図4）。ジャンガリアンハムスターに同じくレプトスピラを接種したが、全く感受性を示さなかったことから、ゴールデンハムスターが特別レプトスピラに対する感受性が高いものと思われる。

診断

血液、脳脊髄液などをEMJH培地、またはKorthof培地などに接種し、30℃、2週～3ヵ月間培養し病原体の分離を行う。10mLの培地に対して、患者から採取した血液1～2滴をベッドサイドで直ちに接種する。初代培養では増殖に時間がかかることもあるが、一方一週間程度でレプトスピラの増殖を見ることもある。第2病週以後では、レプトスピラは尿中に排泄されるが、そのままでは速やかに死滅するので、直ちに遠心し、沈渣をリン酸緩衝生理食塩水に再懸濁し、培地に接種する。

特異抗体は8～10病日に現れはじめ、3～4病週で最高に達する。顕微鏡凝集試験（microscopic agglutination test、MAT）では新鮮菌液（1～2×108／mL）を、急性期、回復期（2病週）のペア血清とマイクロプレート中で反応させ、50%凝集をもって陽性と判定する。ペア血清で4倍以上、単一血清では通常64倍～100倍以上を陽性とする。MATには生きたレプトスピラ培養を必要とすることから、実施できる施設は限られている。また、患者血液、尿等を材料として、鞭毛遺伝子（*flaB*）

などを標的としたポリメラーゼ連鎖反応（PCR）により診断ができる(7)。レプトスピラ症は急性感染症であるため、診断結果を待たずに治療を開始しないと手遅れとなる。

血液、あるいは尿中のレプトスピラを暗視野顕微鏡で検出することは、菌数が必ずしも多くはないことやその他の成分との形態的鑑別が容易ではないことなどから、おすすめできない(16, 17)。

治療

日本ではヒトのレプトスピラ症にはストレプトマイシン1日1～2gずつ、2～4日間筋注が推奨されている。遅くとも第5病日までに治療を開始しないと、十分な効果は期待できない。ゲンタマイシン、トブラマイシン、イセパマイシンでもほぼ同様の効果が期待できる(8)。欧米ではストレプトマイシンの副作用を嫌ってか、ペニシリン、オキシテトラサイクリンが使用されるケースが多いが、長期間大量投与する必要がある。さらにはβラクタム剤により菌体の破壊が起こった結果、LPSなどの菌体成分の漏出が起こり症状の増悪がみられる。これをJarisch-Herxheimer反応という。

予防

全世界で250を越えるレプトスピラ血清型が見つかっているが、日本ではヒトワイル病秋疫混合ワクチンで予防できるのはCopenhageni（Icterohaemorrhagiaeに対しても交差防御）、Autumnalis、Hebdomadis、Australisだけであり、その他の血清型の感染には無効である。動物用としては、イヌ用の血清型IcterohaemorrhagiaeとCanicolaを含むワクチンが使用されている。

レプトスピラの疫学

野生げっ歯類が主要な保有体であることは明らかであるが、近年の患者減少により、レプトスピラ症に注意が払われなくなり、その実態も追究されなくなって久しい。その一方で、世界的交通網の発達、世界規模での物流のなかで、海外からの病原体の侵入はもはや看過できない状況となりつつある。また最近のペットブームで輸入げっ歯類を通じて、これまで日本に存在しない血清型が導入される可能性も危惧されている。我々は海外からの輸入の可能性を危惧して、海外からの貨物船が入港する港で捕獲した野鼠のレプトスピラ保有状況の全国調査、並びに分離株の遺伝学的、血清学的性状解析を行った。3年間で1,101匹の各種野生齧歯類を捕獲し、45株のレプトスピラを分離した（保有率4.1%）。北海道では15%、また長野県八千穂村周辺では9%、鹿児島県の離島である与路島では37%など高頻度レプトスピラを保有する野生齧歯類が棲息する地域が見られた（図5）。また継続的に調査を行っている愛知県名古屋市のマンホールでは毎年コンスタントに数%から7%の保有率を示した。アカネズミ、ヤチネズミ、ドブネズミ、クマネズミ、沖縄ではジャコウネズミなどが主要な保有体となっていた。本土の分離株は血清型Autumnalis、Hebdomadis、IcterohaemorrhagiaeまたはCopenhageni、ほかに血清型が同定できていないが広範囲に見いだされる未同定血清型レプトスピラが高頻度見いだされた。沖縄においては血清型Javanicaが同じく分離されている。このように、今日でもレプトスピラは我々の身近に存在しその感染機会があることが明らかとなった。

最近の事例：輸入動物アメリカモモンガを介したヒトレプトスピラ症例

（症例） 静岡市の動物輸入販売に携わる従業員2名（29歳男性2005年4月22日発病、28歳男性同年6月1日発病）が、相次いで発熱、腰痛及び倦怠感、さらには結膜黄疸、充血、乏尿、血尿を呈し静岡済生会総合病院を受診した。臨床経過、並びに動物の飼育に関わっていたことから、レプトスピラ症を疑いストレプトマイシン2g/日筋注による治療を行った。

表1　顕微鏡凝集試験（MAT）による患者血清診断結果

レプトスピラ抗原株			凝集抗体価	
由来	血清型	株	受診時	回復期
1例目				
基準株	Grippotyphosa	Moskva V	<50	100
アメリカモモンガ由来	Grippotyphosa?	AM1	<50	800
アメリカモモンガ由来	Grippotyphosa?	AM3	<50	800
2例目				
基準株	Grippotyphosa	Moskva V	<50	200
アメリカモモンガ由来	Grippotyphosa?	AM3	<50	200

受診時血清は抗体陰性だったが、回復期血清は100～800倍希釈で陽性となった。
アメリカモモンガ由来株とも反応し、感染源がこの動物である可能性を示唆した。

表2　交差凝集試験による患者、アメリカモモンガ分離株の血清型同定

	アメリカモモンガ分離株					患者分離株		
免疫ウサギ抗血清	AM1	AM2	AM3	AM4	AM7	p5.4	p10.1	p10.2
α-Grippotyphosa	6400	6400	6400	6400	6400	6400	3200	3200
α-Icterohaemorrhagiase	<100	<100	<100	<100	<100	<100	<100	<100
α-Copenhageni	<100	<100	<100	<100	<100	<100	<100	<100
α-Autumnalis	<100	<100	<100	<100	<100	<100	<100	<100
α-Hebdomadis	<100	<100	<100	<100	<100	<100	<100	<100
α-Australis	<100	<100	<100	<100	<100	<100	<100	<100
α-Javanica	<100	<100	<100	<100	<100	<100	<100	<100
α-Castellonis	<100	<100	<100	<100	<100	<100	<100	<100

アメリカモモンガ由来株、患者分離株は、血清型Grippotyphosaに対するウサギ抗血清と特異的に反応したが、その他の血清型に対する抗血清とは反応しなかった。

それぞれ約1週間の治療で回復し、退院となった。

（診断）　両患者の受診時血清はMAT陰性であったが、回復期血清は血清型Grippotyphosaの基準株に対して抗体陽性となり、レプトスピラ症と診断した（表1）。*flaB*を標的としたPCRでも受診時の血清または全血から特異的増幅産物を検出した。また、2例目の患者全血よりレプトスピラの培養に成功した。これらの症例については、感染症法の手続きに従い担当医が患者発生の報告を行った(14)。

（感染源の特定）　この患者発症前に厚生労働科学研究費新興・再興感染症研究事業「輸入動物に由来する新興感染症侵入防止対策に対する研究」研究班（主任研究者　吉川泰弘教授）の研究の一環として、この輸入業者より動物を買い上げ各種病原体の保有調査を実施し、その中でレプトスピラについても検索を行っていた。この輸入業者より買い上げた動物8種（計86匹のうち、アメリカモモンガ（*Glaucomys volans*）の腎臓培養では10匹中5匹よりレプトスピラの増殖を確認した。その他の試験した輸入動物からは検出しなかった。患者、並びにアメリカモモンガ由来の5株は*flaB*、DNAジャイレースBサブユニット遺伝子（*gyrB*）配列解析、およびレプトスピラ全ゲノムの制限酵素断片長多型性解析、各種血清型特異的ウサギ抗血清を用いた交差凝集試験においても同一であることを確認し

た。同じ動物の膀胱凍結試料からも*flaB*-PCRにより増幅産物を検出し、その配列は患者臨床材料、並びに患者分離株、アメリカモモンガ腎臓分離株と完全に一致し、アメリカモモンガを介したレプトスピラ感染事例であったことを証明した（11）。分離株は血清型Grippotyphosaと同定した（表2）。この血清型は米国においては、イヌ、家畜、ヒトのレプトスピラ病起因血清型としてありふれたタイプである（4, 15, 18, 19）。本土には見られないが、沖縄には存在する血清型であったために（13）、我々は免疫抗血清を保有していた。このような幸運が重なり血清型の同定に成功した。我々は過去にも輸入動物のアフリカヤマネがレプトスピラを保有している事実を明らかにしてきたが（12）、今回輸入動物を介したヒトへの感染事例を経験すると共に、その感染源の特定に成功した。

おわりに

平成14年度財務省貿易統計によれば年間190万頭以上もの動物が輸入され、齧歯類は75万頭を超えるという。輸入動物を介した感染症の侵入が危惧されていたが、それが現実のものとなった。2005年9月よりすべての輸入動物に対して衛生証明書の提出が義務づけられるようになったが、昨年同期よりも輸入動物数はむしろ増加しているという。今後も輸入動物を介した病原微生物の侵入に対して監視を続けていく必要があろう。

追記

本研究の一部は平成15～17年度厚生労働省厚生科学研究費新興再興感染症研究事業（主任研究者　増澤俊幸）の助成のもと実施された。アメリカモモンガ感染事例は、麻布大学　宇根有美先生、静岡済生会病院　竹内隆浩先生、塚越敬子先生、国立感染症研究所　川端寛樹先生、小泉信夫先生、東京大学　吉川泰弘先生のご協力のもと平成15～17年度厚生労働省厚生科学研究費新興再興感染症研究事業（主任研究者　東京大学　吉川泰弘）の支援のもと感染源の特定に至った。

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Novel Approach to Designing Primers for Identification and Distinction of the Human Pathogenic Fungi *Coccidioides immitis* and *Coccidioides posadasii* by PCR Amplification

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We developed a pair of primers that specifically identifies *Coccidioides* species, etiologic agents of the human fungal disease coccidioidomycosis. These primers could be used for distinguishing *Coccidioides immitis* and *Coccidioides posadasii* by simply comparing the amplicon sizes on an agarose gel.

Coccidioidomycosis, a fungal respiratory disease of humans caused by *Coccidioides* species, is endemic to arid areas of the Americas. Two species of *Coccidioides* are now recognized, whereas until recently, coccidioidomycosis was attributed to only one species, *Coccidioides immitis* (4). *Coccidioides posadasii*, formerly known as the non-California *C. immitis* strain, is found mainly in Texas, Arizona, and regions of endemicity outside of the United States, whereas *C. immitis* is found primarily in the Central Valley of California. These two species can be divided based on single-nucleotide polymorphisms and the size of microsatellites (3, 4), although the colony morphologies, growth rates, and clinical presentations are almost identical. Because identification of *Coccidioides* spp. carries with it a great deal of risk, molecular diagnosis without culturing has long been expected. Many researchers have explored nucleic acid detection for the diagnosis of coccidioidomycosis (2, 7–9, 11, 12).

For isolates of *C. immitis* and *C. posadasii*, listed in Table 1, the same DNA samples previously described by Sano et al. (10) were used. For non-*Coccidioides* fungal isolates, listed in Table 2, DNA isolation was performed using a DEXPAT DNA extraction kit (Takara Bio Inc., Japan). PCR was performed with approximately 10 ng of extracted DNA in a 20-μl reaction volume consisting of LA *Taq* buffer II (Mg^{2+} plus) (Takara Bio Inc.), 200 μM deoxynucleoside triphosphates, 2.5 U of ExTaq DNA polymerase (Takara Bio Inc.), and 10 pmol of each primer. One cycle at 94°C for 3 min followed by 35 cycles at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 45 s with a final extension step at 72°C for 3 min was performed in a PTC-200 DNA Engine thermal cycler (Bio-Rad). The amplified DNA must be handled carefully in order to avoid amplicon contamination. The universal fungal primers ITS1 and ITS4 were used in all DNA samples to verify the efficiency of the test and to ensure that there was no PCR inhibition in the DNA samples (8). Ten microliters of each PCR product was electrophoresed through 2% agarose gel and visualized with a UV light after ethidium bromide or SYBR Safe (Invitrogen) staining. The PCR products were purified from gel with a NucleoSpin Extract II kit (Macherey-Nagel, Germany). Nucleotide sequences were determined using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) and an ABI 3130 genetic analyzer (Applied Biosystems).

Empirically, the construction of diagnostic primers is based on a nucleotide sequence encoding conserved enzymes or rRNA. However, we left the matter to chance: we repeated the primer construction based on randomly selected regions and verification by actual PCR experiments. In step 1, we obtained a text file containing the *C. immitis* genome sequence (http://www.broad.mit.edu/annotation/fungi/coccidioides_immitis/). A nucleotide sequence corresponding to the 240- to 720-bp length was randomly selected from the genome database file. We designed 20-mer forward and reverse primers, which were expected to amplify the randomly selected region. In step 2, we examined whether these two primers could amplify DNA fragments of the anticipated size from *Coccidioides* spp. In step 3, we tested whether the primers that could amplify *Coccidioides* DNA in step 2 were unable to amplify *Candida albicans* and *Aspergillus fumigatus* DNA. By repeating these steps on 64 selected regions, we nominated one pair of primers for more detailed examination. Since this experimental primer design led to our successful product, mentioned below, this strategy should be a powerful method for the development of diagnostic primers.

The selected primers were Coi9-1F (5′-TACGGTGTAATC CCGATACA-3′) and Coi9-1R (5′-GGTCTGAATGATCTG ACGCA-3′). The selected primer set was constructed to amplify a 720-bp amplicon that corresponds to nucleotide position 660313 to 661032 of *C. immitis* contig 2.2 (accession number AAEC02000002). Nineteen isolates of *Coccidioides* spp. were examined for the developed primers (Table 1). The PCR system with the specific primer pairs was able to amplify the DNA fragment of the expected size from DNAs of *C. immitis* (Fig. 1). For specificity testing, 137 isolates of 52 fungal species were examined (Table 2). As a result of PCR using DNAs from these fungi, the primers were proved not to cross-amplify with major pathogenic fungi and related ones, such as *Arthrographis kalrae*, *Chrysosporium* spp., *Geotrichum candidum*, *Malbranchea* spp.,

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TABLE 1. *Coccidioides* isolates used in this study

Lane no. in Fig. 1	Species	IFM no.[a]	Origin
1	*C. posadasii*	4935	Japan
2	*C. posadasii*	4945	Japan
3	*C. posadasii*	45809	United States
4	*C. posadasii*	45810	United States
5	*C. posadasii*	45811	United States
6	*C. posadasii*	45812	United States
7	*C. posadasii*	45813	United States
8	*C. immitis*	45815	United States
9	*C. immitis*	45816	United States
10	*C. posadasii*	45817	United States
11	*C. immitis*	46868	Japan
12	*C. immitis*	50992	United States
13	*C. posadasii*	50993	Japan
14	*C. posadasii*	50994	Japan
15	*C. immitis*	50995	Japan
16	*C. posadasii*	51112	Japan
17	*C. posadasii*	54194	Japan
18	*C. posadasii*	54195	Japan
19	*C. posadasii*	54196	Japan

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TABLE 2. Fungal species used as negative controls in this study

Species	No. of tested isolates
Absidia sp.	1
Alternaria sp.	1
Apinisia spp.	2
Arthroderma spp.	8
Arthrographis spp.	4
Aspergillus spp.	5
Auxarthron sp.	1
Basidiobolus sp.	1
Blastomyces sp.	1
Candida spp.	8
Chrysosporium spp.	4
Cladophialophora spp.	2
Cokeromyces sp.	1
Conidiobolus sp.	1
Cryptococcus spp.	5
Cunninghamella sp.	1
Emmonsia spp.	3
Epidermophyton sp.	1
Exophiala spp.	4
Fonsecaea sp.	1
Fusarium spp.	2
Geotrichum sp.	1
Gymnoascoideus spp.	3
Gymnoascus spp.	5
Histoplasma spp.	6
Hortaea sp.	1
Malassezia spp.	2
Malbranchea spp.	17
Microsporum spp.	2
Mortierella sp.	1
Mucor spp.	2
Neosartorya sp.	1
Paecilomyces spp.	2
Paracoccidioides spp.	6
Penicillium spp.	7
Phanerochaete spp.	2
Phialophora spp.	2
Prototheca sp.	1
Pseudallescheria sp.	1
Rhinocladiella sp.	1
Rhizomucor sp.	1
Rhizopus spp.	3
Scedosporium sp.	1
Schizophyllum sp.	2
Scopulariopsis sp.	1
Sporothrix sp.	1
Syncephalastrum sp.	1
Trichophyton spp.	3
Trichosporon sp.	1
Uncinocarpus sp.	1
Veronaea sp.	1
Zygorhynchus sp.	1

Paracoccidioides brasiliensis, and *Trichosporon asahii*. The *Coccidioides* diagnostics based on a proline-rich antigen (2, 6) or internal transcribed spacer region (5, 7) have been reported so far. However, Bialek (1) has pointed out that the possibility of cross-amplification of human and murine DNA by the ITS primers (7) was not excluded. Since no amplification of human DNA by the primers in this report was detected (data not shown), we have developed a useful PCR system applicable for use in clinical diagnosis.

Surprisingly, two different mobilities of the DNA fragment were observed (Fig. 1) when the primers were tested for all *Coccidioides* spp. DNA available in Japan (Table 1). The DNA fragment amplified from *C. posadasii* was obviously shorter than that from *C. immitis*. Nucleotide sequence analysis of the amplified DNA revealed that the amplicon from *C. posadasii* had a contiguous deletion of 86 bp compared to that from *C. immitis* (Fig. 2). Therefore, such a large deletion contributed to the convenient distinction of two very close species, *C. immitis* and *C. posadasii*, which had previously been difficult to distinguish. In actual distinction on an agarose gel, standard amplicons of *C. immitis* and *C. posadasii* should be run as controls

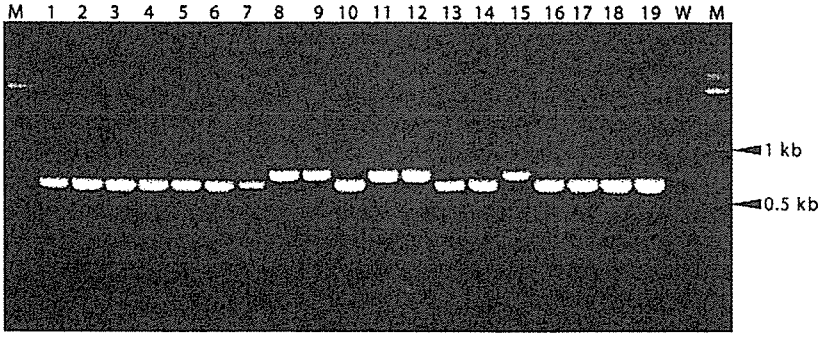

FIG. 1. PCR amplification of coccidioidal DNAs. Lanes M, DNA molecular weight marker used to estimate product size; lane W, distilled water used as a negative control; lanes 1 to 7, 10, 13, 14, and 16 to 19, *C. posadasii*; lanes 8, 9, 11, 12, and 15, *C. immitis*. The exact description of *Coccidioides* spp. is in Table 1.

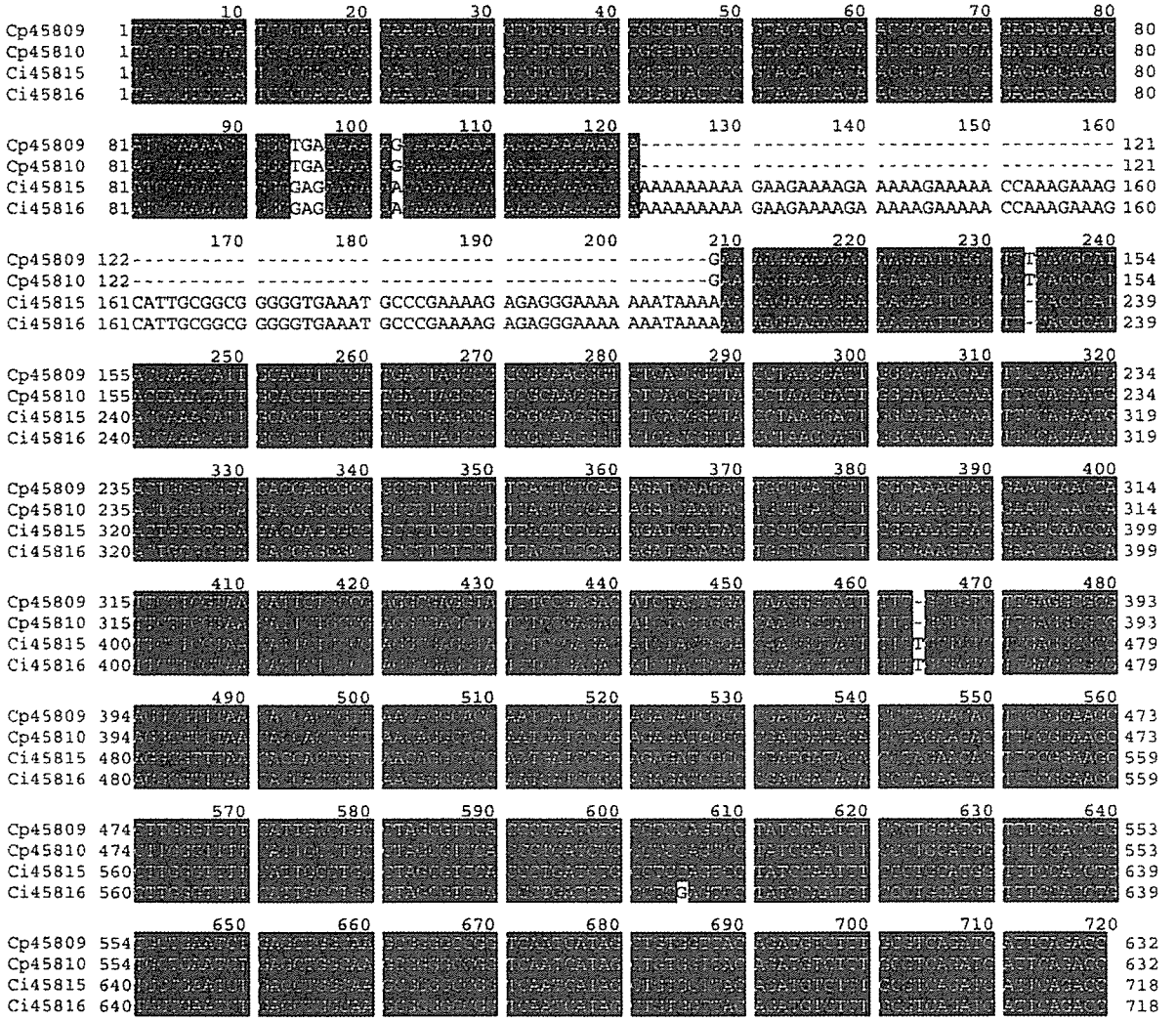

FIG. 2. Nucleotide sequence alignment of DNA fragments amplified with Coi9-1F and Coi9-1R primers between *C. posadasii* IFM45809 (Cp45809) and IFM45810 (Cp45810) and *C. immitis* IFM45815 (Ci45815) and IFM45816 (Ci45816).

because of the close proximity of the two bands. Two methods for differentiating *C. immitis* and *C. posadasii* are currently being used: the lengths of the microsatellite loci and the single-nucleotide polymorphisms within several enzymes (3). Both methods require highly skilled molecular biological techniques at a relatively high cost. The PCR system with the primers we developed might facilitate operation and provide high-throughput handling. Thus, this will provide high value in epidemiology, such as tracing the route of laboratory-acquired infection or analyzing a pandemic that might occur in the future. Whether these primers can be clinically applied remains to be seen. Further development will contribute to the early diagnosis of coccidioidomycosis.

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Short Report

Reexamination of *Coccidioides* spp. Reserved in the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Based on a Multiple Gene Analysis

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Abstract

The Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University is the only organization in Japan to possess a series isolates of *Coccidioides* spp., which are the most virulent pathogenic fungi and which are treated as biosafety level 3 microorganisms. Recently, the genus *Coccidioides* has been classified into two species, *C. immitis* and *C. posadasii*, based on their endemic areas and genotyping; the former species is endemic to the state of California, and the latter is endemic to other parts of North and South America. We reevaluated 19 isolates of *Coccidioides immitis* stored in our center using a multiple gene analysis. Five isolates were identified as *C. immitis* and 14 as *C. posadasii*. Their sequence information in GenBank will help to identify the two genospecies of *Coccidioides* spp.

Key words: *Coccidioides immitis*, *Coccidioides posadasii*, multiple gene analysis

Introduction

Coccidioides spp., the causative agents of coccidioidomycosis are the most virulent among pathogenic fungi. Coccidioidomycosis is treated as an imported fungal infection in Japan because almost all the patients were assumed to have gotten the infection in the United States or Mexico. It causes pneumonia and sometimes fatal systemic infection in healthy subjects, and is considered as a category-4 infection by the Ministry of Health, Labor, and Welfare of Japan. Clinicians who see patients with category-4 infections are required to report them to a local public health center[1].

The habitats of *Coccidioides* spp. are desert areas in North and South America. In Arizona, an endemic area, more than 2,000 new cases per year are reported and more than 100,000 individuals are infected with the fungus without symptoms[2]. In Japan, more than 45 cases had been recorded as of November 2005 (http://www.pf.chiba-u.ac.jp/). Most of the Japanese patients were infected in endemic areas to which they had traveled or resided, except for 1 case caused by contact with crude cotton imported from the United States. We could not determine the place of infection for some patients who had traveled to several endemic areas in The United States and Mexico.

The disease is caused by inhalation of arthroconidia of the fungi. The fungi grow as mycelia in spring and produce arthroconidia. They are easily spread into the air by the wind, by new construction activities, and by excavations. The clinical isolates of *Coccidioides* spp. on slants or plates are also easily diffused into the air and are apt to cause infections in laboratories. The culturing and identification of the fungi by mycological examination require at least 2 weeks for experts with special training[1].

C. immitis has been treated as a single species as the causative agent of coccidioidomycosis. Since 2002, *C. immitis* has been classified into

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two species, *C. immitis* and *C. posadasii*, based on the endemic areas and genotypes; the former species is endemic to the state of California, while the latter is endemic to other parts of North and South America[3]. In 1997, Koufopanou *et al.* tried to reclassify the species into two geographic types, a California type and an outside-California type, based on the combination of five gene analogues: chitin synthase, dioxygenase (DO), orotidine decarboxylase, serine proteinase (SP), and chitinase (CT)[4]. Fisher *et al.* created the two genospecies taxa based on the multiple gene analysis of microsatellites[3]. They also suggested a convenient method for the differentiation of *Coccidioides* spp. by a multiple gene analysis using a combination of three gene sequences: DO, SP, and CT[5]. The sequence data obtained from their investigations were not released to GenBank completely, because of patenting and bioterrorism crisis-management programs.

The present study aimed to reevaluate 19 strains formerly identified as *C. immitis* in our center following the criteria for genospecies proposed by Fisher *et al.* in 2002, to release their sequence data for public convenience, and to clarify the place of infection for Japanese patients. In addition to the genes used by Fisher *et al.*[3], we analyzed the internal transcribed spacer (ITS) 1-5.8S-ITS 2 and the D1/D2 regions of ribosomal RNA genes that are generally used as phylogenetic markers[6, 7] as well as a partial sequence of the urease gene (URE), which codes the virulence factor of pathogenic fungi[8]. The present study also tried to find specific genes to classify *Coccidioides* spp. from the genes named above.

Materials and Methods

Coccidioides spp. strains examined are shown in Table 1. They were formerly identified as *C. immitis* by the morphology observed through the test tubes, by the patient's history, or by identifications in the original country.

The fungi were cultured at 37°C for 2 weeks on potato dextrose agar (Difco, Franklin Lakes, NJ, USA) slants with a silicon plug. Then, a needle with a syringe containing 85% ethanol was pierced the plug and ethanol was slowly injected until the plug was immersed. The point of needle insertion into the plug was covered with a mass of cotton soaked with 70% ethanol. The fungal mass was fixed by a final concentration of approximately 70% ethanol for 48 hours at room temperature. A loop of fungal mass was spread on a PDA plate and cultured at 37°C for 7 days to check its survival. The next step was started after we confirmed that the fungus had no sprouts. The above procedures were done in a special laboratory for level 3 biohazard pathogens.

DNA was extracted with a DEXPAT® Kit (TaKaRa, Ohtsu, Japan) with a modification of the manufacturer's protocol. Approximately 100 μl of the fungal mass was transferred to a sterilized microtube (1.5 ml), homogenized with 0.5 ml of DEXPAT® solution by a plastic pestle. The mixture was incubated at 100°C for 10 min and centrifuged at 12,000 rpm (13,201 g) for another 10 min. The supernatant was used

Table 1. *Coccidioides* spp. isolates and their accession numbers

Isolate					Gene (length)				
IFM No.	Strain	Identification	Origin	Country	Deoxygenase (636)	Serine proteinase (646)	Chitinase (487)	Urease (536)	rRNA (1251)
4935	Nagoya	*C. posadasii*	Human case	Japan	AB232864	AB232726	AB232745	AB232707	AB232883
4945	Ohashi	*C. posadasii*	Human case	Japan	AB232865	AB232727	AB232746	AB232708	AB232884
45809	Silviera (SAP2)	*C. posadasii*	Animal passage II	USA	AB232866	AB232728	AB232747	AB232709	AB232885
45810	Silveira (SAP3)	*C. posadasii*	Animal passage III	USA	AB232867	AB232729	AB232748	AB232710	AB232886
45811	Arizona	*C. posadasii*	Human case	USA	AB232868	AB232730	AB232749	AB232711	AB232887
45812	San Antonio	*C. posadasii*	Human case	USA	AB232869	AB232731	AB232750	AB232712	AB232888
45813	New York	*C. posadasii*	Human case	USA	AB232870	AB232732	AB232751	AB232713	AB232889
45815	91-48	*C. immitis*	Human case	USA	AB232871	AB232733	AB232752	AB232714	AB232890
45816	91-153	*C. immitis*	Human case	USA	AB232872	AB232734	AB232753	AB232715	AB232891
45817	Nicols	*C. posadasii*	Human case	USA	AB232873	AB232735	AB232754	AB232716	AB232892
46868	Yokohama	*C. immitis*	Human case	Japan	AB232874	AB232736	AB232755	AB232717	AB232893
50992	90-242	*C. immitis*	Human case	USA	AB232875	AB232737	AB232756	AB232718	AB232894
50993	Kanazawa	*C. posadasii*	Human case	Japan	AB232876	AB232738	AB232757	AB232719	AB232895
50994	Toranomon	*C. posadasii*	Human case	Japan	AB232877	AB232739	AB232758	AB232720	AB232896
50995	Handai	*C. immitis*	Human case	Japan	AB232878	AB232740	AB232759	AB232721	AB232897
51112	Himeji	*C. posadasii*	Human case	Japan	AB232879	AB232741	AB232760	AB232722	AB232898
54194	Nagano	*C. posadasii*	Human case	Japan	AB232880	AB232742	AB232761	AB232723	AB232899
54195	Fukunaga	*C. posadasii*	Human case	Japan	AB232881	AB232743	AB232762	AB232724	AB232900
54196	Chiba	*C. posadasii*	Human case	Japan	AB232882	AB232744	AB232763	AB232725	AB232901

as the DNA sample[9]. DNA extract (2.5 μl), Ready-to-Go beads (Amersham Pharmacia, Tokyo, Japan), 20 μl of distilled water, and 2.5 μl of 10 pM of each primer for DO, SP, CT, and rRNA genes[4-7] were mixed, and polymerase chain reactions (PCR) were processed. PCR products were separated by electrophoresis on 1.0% agarose gels in 1 × TBE buffer (0.04 M Tris-boric acid, 0.001 M EDTA pH 8.0) and visualized by ethidium bromide staining. PCR products were purified with a PCR purification kit (QIAquick®, Qiagen) and labeled using BigDye® terminator Ver. 1.1 (Applied Biosystems, Foster City, CA, USA) by the manufacturer's protocol.

The partial sequence of the urease gene (URE) was amplified with a primer set (CIS2410-2434: CGG GTA TTT ACA AGG CTG ATA TTG G and CIAS2945-2922: GAA GCC AGA TTC GTT CAG GGT GTC) designed from the *C. immitis* urease gene sequence deposited in GenBank under accession number U81509[8]. The PCR conditions for URE were as follows: the reaction mixture was subjected to 1 cycle of denaturation at 95°C for 4 min, 30 cycles of amplification at 94°C for 1 min, at 50°C for 1 min, and at 72°C for 2 min, and a final extension cycle at 72°C for 10 min with a PCR Thermal Cycler MP (TaKaRa).

The combined data set for phylogenic study was performed in *Fusarium* spp.[10]. The present study applied above analysis for alignment of combined sequences consisted of at least 1,769 base pairs obtained from the DO, SP, and CT genes using CLUSTAL X (Version 1.8)[11]. An unrooted tree was constructed using Njplot (http://pbil.univ-lyon1.fr/software/njplot.html)[12]. The trees were also constructed based on each gene alone. Consistencies in clade formation between the unrooted trees based on each gene and the combined one were compared.

The genospecies of *Coccidioides* were determined based on the location of the clade. Strains located in the clade involving IFM 50995 derived from a Japanese patient whose infection was suspected of having occurred in Bakersfield, California[13] was identified as *Coccidioides immitis*, and those in the clade with IFM 45811 and IFM 45812 originating in the state of Arizona were identified as *Coccidioides posadasii*, respectively.

Results and Discussion

The accession numbers of the genes, the lengths of the sequences, and the identification based on the cluster analysis with the three-gene combination are shown in Table 1. The unrooted tree based on a combination of three genes is shown in Fig. 1. Five isolates of *Coccidioides* spp. in our center were identified as

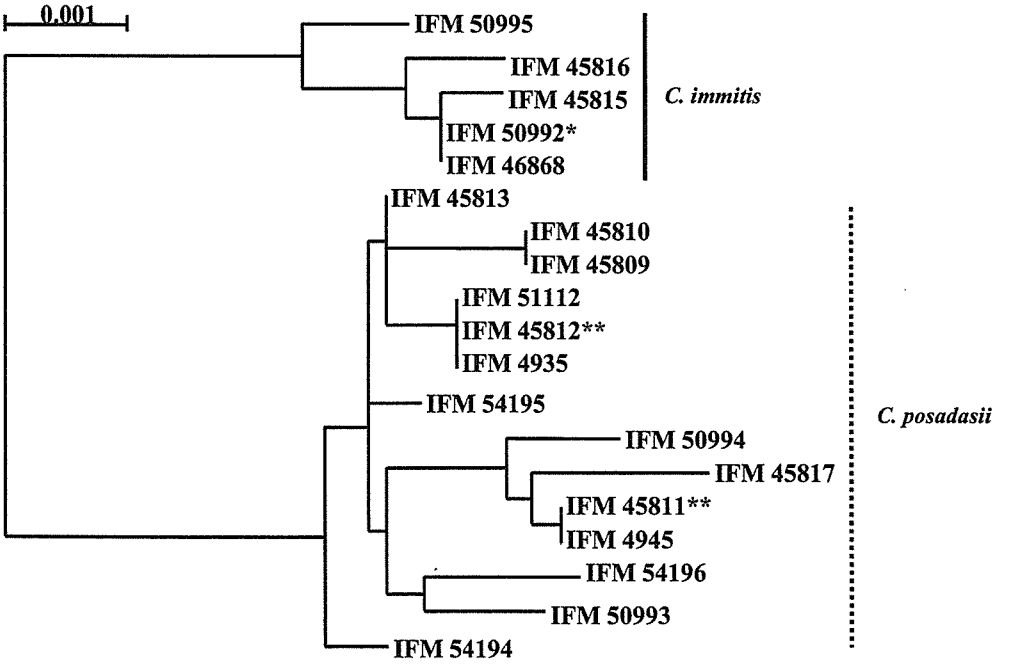

Fig. 1. Unrooted tree based on a combination of three gene sequences-dioxygenase (DO), serine proteinase (SP), and chitinase (CT) consisting of 1769 base pairs constructed by the neighbor-joining method. The scale bar represents a difference corresponding to 0.001 (0.1%). Isolates identified as *C. immitis* are in the upper clade and those as *C. posadasii* are in the lower one. *: isolate used as *C. immitis* standard; **: isolate used as *C. posadasii* standard.

C. immitis and 14 as *C. posadasii*. The data confirmed that 2 of the Japanese cases were caused by *C. immitis*.

Unrooted trees based on individual DO, SP, and URE genes were consistent with the combination of three genes (DO, SP, and CT) and could separate two clades, while those of CT and rRNA genes could not (data not shown). The homology among isolates was more than 99% in all genes evaluated.

The GenBank database was not sufficient for genetic identification of *Coccidioides* spp. There was one sequence of DO on *C. posadasii* (L38493), four sequences of SP on *C. immitis* (S77562, AJ408857, AJ408861, and M81863) and four sequences on *C. posadasii* (AJ408858, AJ408859, AJ408860, and X63114), three sequences of CT on *C. posadasii* (L41663, U51271, and U60806), one sequence of URE on *C. posadasii* (U81509), two sequences of the ITS region of rRNA on *C. posadasii* (CIU18360, X94142), and two sequences of the D1/D2 region of rRNA on *C. immitis* (AY176713, AB040702). The gene sequences of the species used for the present study are helpful for genetic identification. Cluster analysis in the present study with a combination of these data found an inconsistency on the SP gene. The sequence AJ408857, derived from *C. immitis* in the GenBank database, was located on the cluster of *C. posadasii*.

Thus far, two genes, DO and URE, might be useful for identifying genospecies of *Coccidioides* spp. alone. Such an analysis will allow speculation on where infection occured, while both the ITS and D1/D2 regions of rRNA genes, which are representative genetic markers for classifying and identifying fungal species[6, 7], were incompatible with the unrooted tree based on the cluster analysis by combination of the three genes. Identification based on ribosomal RNA genes could not identify the species of *Coccidioides* because of strongly similar identity among strains according to the criteria proposed by Kurtzman and Robnett[7].

The inter species differences between *C. immitis* and *C. posadasii* linked both geographic distribution and virulence[5]. However, clinical isolates of both *C. immitis* and *C. posadasii* in Japan should be regarded as the most virulent fungal species. We should keep in mind that the clinical isolates of *Coccidioides* spp. should be handled in accordance with biohazard regulations at a bio-safety level 3 laboratory.

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Short communication

Rapid identification of *Ochroconis gallopava* by a loop-mediated isothermal amplification (LAMP) method

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Abstract

Ochroconis gallopava is a species of dematiaceous fungi recognized as a causative agent of zoonotic and emerging fungal infections. It affects the central nervous system and respiratory tracts of humans, birds and cats. We designed *O. gallopava* species-specific primer sets to aid in its identification by a loop-mediated isothermal amplification (LAMP) method based on the D1/D2 domain of the LSU rDNA sequence. The LAMP method successfully detected the gene from both fungal DNA and experimentally infected brains and spleens of mice and will be helpful in the diagnosis of *O. gallopava* infection.

Keywords: D1/D2 domain; Loop-mediated isothermal amplification (LAMP); *Ochroconis gallopava*; Phaeohyphomycosis; Zoonosis

1. Introduction

Ochroconis gallopava is a species of dematiaceous fungi recognized as a causative agent of zoonotic and emerging fungal infections (de Hoog et al., 2000). More than 30 human cases have been reported (Fukushima et al., 2005). The pathogen has caused outbreaks in poultry and wild birds, and a few cases in domestic cats (Kralovic and Rhodes, 1995). Environmental isolates of *O. gallopava* have also been found under low-pH and thermal conditions, such as in coal waste piles and hot springs, sewage from nuclear power plants, and broiler-house litter (Kralovic and Rhodes, 1995).

The disease in birds resembles the highly pathogenic H5N1 avian influenza (HPA) involving the central nervous system and respiratory tract. Therefore, a simple and rapid method of diagnosing *O. gallopava* infection is eagerly awaited because diagnosis by the isolation and identification of the fungus using mycological techniques is time-consuming and requires expertise.

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We recently developed the species-specific primer set of a highly pathogenic fungal species, *Paracoccidioides brasiliensis*, for use in a loop-mediated isothermal amplification (LAMP) method (Endo et al., 2004). The present study applied this method to the diagnosis of *O. gallopava* infection.

2. Materials and methods

2.1. Isolates

The species names *Diplorhinotrichum gallopavum*, *Dactylaria gallopava*, *O. gallopava*, *Dactylaria constricta* var. *galopava* and *Ochroconis gallopavum* are treated herein as *O. gallopava*, as proposed by de Hoog et al. (2000).

The *O. gallopava* isolates and related dematiaceous fungal species, and their genetic profiles of the divergent region of the D1/D2 domain of the large subunit of the rRNA gene (D1/D2 LSU rDNA) evaluated in the present study are shown in Table 1. Eight clinically important fungal species, *Aspergillus fumigatus* (IFM 40821), *Blastomyces dermatitidis* (IFM 41316), *Candida albicans* (IFM 5740), *Coccidioides immitis* sensu lato (IFM 50993), *Cryptococcus neoformans* (IFM 5830), *Histoplasma capsulatum* (IFM 41329), *Penicillium marneffei* (IFM 41708) and *Sporothrix schenckii* (IFM 47068), were also tested as negative controls.

2.2. Sequences for D1/D2 LSU rDNA

Fungal DNAs were extracted from cultures incubated on potato dextrose agar (PDA) slants (Difco, Franklin Lakes, NJ, USA) at 25 °C for 1–2 weeks. DNA was extracted with a DEXPAT® Kit (TaKaRa, Ohtsu, Japan) following the manufacturer's protocol with slight modification. Approximately 100 μl of fungal mass was transferred to a sterilized microtube (1.5 ml), and homogenized with 0.5 ml of DEXPAT® solution by a plastic pestle. The mixture was incubated at 100 °C for 10 min and centrifuged at 12,000 rpm (13,201 × *g*) for 10 min. The supernatant was used as the DNA sample (Sharmin et al., 2002). The sequence of D1/D2 LSU rDNA was processed by a standard method (Kurtzman and Robnett, 1997).

2.3. Species-specific PCR primer set for Ochroconis gallopava

A species-specific polymerase chain reaction (PCR) primer set for *O. gallopava* was designed based on the sequence of D1/D2 LSU rDNA of *O. gallopava* (accession number AB125281 in GenBank) with a comparison of 22 species of dematiaceous fungi obtained from the present study and from GenBank (*Cladophialophora bantiana*, AB100676; *Exophiala jeanselmei*, AB100664; *Exophiala spinifera*, AB100673; *Fonsecaea pedrosoi*, AB100632; *Phialophora verrucosa*, AB100610; *Rhinocladiella atrovirens*, AB091215). The primer sequences (Sigma-Genosys Japan, Ishikari, Hokkaido, Japan) were the following: OgF3: 5′-AGG GAG TCT CGG GTT AAG GG-3′ encoding from the 391st to the 410th, and OgB3: 5′-CAT TCC CTT CGT CTT TGT CC-3′ corresponding to the complementary sequence from the 718th to the 740th of AB125281.

PCR was carried out with the species-specific primer set under the following conditions. Approximately 20–40 ng of template DNA in 2.5 μl, one Ready-To-Go™ PCR bead (Amersham Pharmacia, Tokyo, Japan) and 10 pmol of primers OgF3 and OgB3 in 25 μl of total volume was subjected to an initial denaturing step of 4 min at 95 °C, 30 cycles of 1 min at 94 °C for DNA denaturation, 90 s at 58 °C for primer annealing, 2 min at 72 °C for primer extension, a final extension of 10 min at 72 °C, and was then maintained at 4 °C until electrophoresis. The PCR products from *O. gallopava* were sequenced by a method (Sharmin et al., 2002). The detection limits of the species-specific PCR for fungal DNA were evaluated in a serial dilution of DNA.

2.4. Loop-mediated isothermal amplification (LAMP) method

The specific primer sets for the LAMP method were as follows: OgF3, OgB3, FIP; 5′-ACT CGA CTC GTC GAA GGG GCA GAG GGT GAG AGT CCC GT-3′ designed from the forward sequence of 425th to 445th and the complementary sequence of 464th to 485th, and BIP; 5′-ACT GGC CAG AGA CCG ATA GCG TGA CTC TCT TTT CAA AGT GC-3′ designed from the forward sequence from 648th to 668th and the complementary sequence from 691st to 712nd of

Table 1
Strains examined

Species	IFM number	Remarks	Country	Source	Sequence length (identity, %)[a]	Accession number
Ochroconis gallopava	IFM 41473	NHL2917	Japan	Subcutaneous abscess	615 (99.7)	AB125280
	IFM 52602		Japan	Bulla infection with pneumoconiosis	615 (99.7)	AB125281
	IFM 52603	CDC B-5637 (=CBS 863.95)	USA	HIV-positive organ recipient	615 (99.7)	AB125282
	IFM 52604	CDC B-5346	USA	Brain abscess of a domestic cat	615 (99.7)	AB125283
	IFM 52605	CDC B-4224 (=ATCC 60633)	USA	Brain abscess of a patient with leukemia and diabetes	615 (99.7)	AB125284
	IFM 52663	CBS 437.64^T (=CDC B-579, CDC 45-492-62, ATCC 16027, MUCL 6683, UAMH 5417)	USA	Brain abscess of a turkey (*Meleagridis gallopavo*)	615 (99.7)	AB125285
	IFM 52664	CBS 866.95 (=CDC B-4767)	USA	Brain of a leukemic patient	615 (99.7)	AB125286
	IFM 52846	UAMH 5413[b] (=DMD381)	USA	Bronchial washing	615 (99.7)	AB161047
	IFM 52847	UAMH 5415[b] (=DMD 409, ATCC 48579, PLM 1478)	USA	Pleural lesion of a domestic cat	615 (99.7)	AB161048
	IFM 52848	UAMH 5418[b] (=DMD 412, ATCC 26841)	USA	Coal pile (coal mine waste)	615 (99.7)	AB161049
	IFM 52849	UAMH 6552[b] (=CDC-B-4872, CBS 865.95)	South Africa	Sputum	615 (99.7)	AB161050
	IFM 52850	UAMH 7486[b]	New Zealand	Lung of an antipodean parakeet (*Cyanoramphus unicolor*)	615 (99.7)	AB161051
	IFM 52851	UAMH 7573[b]	Canada	Pulp sample with pink slime	615 (99.7)	AB161052
	IFM 52852	UAMH 6550[b] (=CDC-B-4682, CBS 867.95)	USA	Sputum	615 (99.7)	AB161053
	IFM 52853	UAMH 6553[b] (=CDC-B-4873)	South Africa	Sputum of amine worker	615 (99.7)	AB161054
	IFM 52854	UAMH 7355[b] (=CDC-B-5304)	USA	Lung and brain of a heart transplant patient	615 (99.7)	AB161055
	IFM 52855	UAMH 7523[b]	Canada	Bronchial washing	615 (99.7)	AB161056
	IFM 52856	UAMH 8240[b]	New Zealand	Sputum	615 (99.7)	AB161057
	IFM 52857	UAMH 8535[b]	USA	Lung	615 (99.7)	AB161058
	IFM 52974	CBS 166.85	France		615 (99.7)	AB161059
	IFM 52975	CBS 547.81	New Zealand		615 (99.7)	AB161060
	IFM 52976	CBS 265.97	Australia	Brain	615 (99.7)	AB161061
	IFM 52977	CBS 100437 (=ATCC 48169, IMI 241149)	UK	Encephalitis of broiler chicken	615 (99.7)	AB161062
Alternaria alternata	IFM 53880		Japan	Skin of a dog	614 (79.2)	AB192875
Arthrobotrys javanica	IFM 52658	CBS 534.63^T (=IMI 108725)	Indonesia	Soil	612 (80.1)	AB161069
Bipolaris spicifera	IFM 41474	CBS 246.62 (=IMI 091972)	UK	Cotton pulp	614 (79.4)	AB161071

Table 1 (*Continued*)

Species	IFM number	Remarks	Country	Source	Sequence length (identity, %)[a]	Accession number
Cladophialophora carrionii	IFM 4808	ATCC 16264 (=CDC A-835, CBS 160.54, MUCL 40053, DCU 310)	Australia	Chromoblastomycosis	613 (80.7)	AB161076
Curvularia geniculata	IFM 41459	IFO 7407	unknown	Unknown	614 (79.1)	AB161072
Curvularia lunata	IFM 41460	IFO 30883	Japan	Leaf (*Sorgum bicolor*)	614 (79.1)	AB161073
Curvularia senegalensis	IFM 52187		Brazil	Environment	614 (79.2)	AB161074
Exophiala alcalophila	IFM 4957		Japan	House bath	616 (81.9)	AB192876
Exophiala dermatitidis	IFM 4827	KUM 1184^T (=IFO 6421, CBS 207.35, ATCC 28869, Duke Univ 2400, IMI 093967, LSHTM 1135, NCPF 2422, UAMH 3967)	Japan	Chromomycosis	617 (81.4)	AB161077
Exophiala moniliae	IFM 4966		Japan	Public bath	619 (80.8)	AB161078
Ochroconis constricta	IFM 52654	CBS 202.27 (=MUCL 9471)	USA	Soil	619 (81.1)	AB161063
Ochroconis gamsii	IFM 52653	CBS 239.78	Sri Lanka	Leaf (*Caryota plumosa*)	619 (89.4)	AB161064
Ochroconis humicola	IFM 52655	CBS 780.83	Japan	Leaf (*Podocarpus*sp.)	613 (89.0)	AB161068
Ochroconis tshawytschae	IFM 41597		Japan	Fish disease	615 (89.5)	AB161067
	IFM 52657	CBS 100438^T (=ATCC 9915)	USA	Fish	615 (89.3)	AB161066
Scolecobasidium terreum	IFM 52656	CBS 203.27^T (=MUCL 9563)	USA	Soil	608 (78.4)	AB161065

ATCC, American type culture collection, Rockville, MD, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; DCU, Department of Dermatology, School of Medicine, Chiba University, Chiba, Japan; IFM, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan; IFO, Institute for Fermentation, Osaka, Japan; KUM, Department of Dermatology, School of Medicine, Kanazawa University, Kanazawa, Ishikawa, Japan.

[a] Homology was compared with the sequences of *O. gallopava* isolates.

[b] Received as *O. gallopavum*.

AB125281. One microliter of DNA template and 5 pmol each of OgF3 and OgB3, and 40 pmol each of FIP and BIP primers were mixed with 12.5 μl of a reaction mixture in a kit (Loop AMP, Eiken, Tokyo, Japan) in a final volume of 25.0 μl. The DNA mixtures were incubated at 63 °C for 60 min and 80 °C for 2 min. The amplified DNAs were detected by electrophoresing in a 1.0% agarose gel stained with ethidium bromide. The detection limits of the LAMP method were evaluated with serial dilutions of a fungal DNA of *O. gallopava*.

2.5. Experimental infection of O. gallopava

The conidia were collected from PDA slants cultured at 25 °C for 4 weeks. Approximately 10 ml of sterile saline was poured on the surface of a slant, and the fungal suspension was collected after gentle scraping with a plastic sterile loop. Large mycelial fragments were removed by passing through a glass filter, after which the samples were washed three times with sterile saline and the concentration was adjusted to 5×10^6/ml.

Five male ddY mice aged 6 weeks (SLC, Shizuoka, Japan) were treated subcutaneously with 150 mg/kg of corticosteroid (Hydrocortone®, Banyu Pharmaceutical Co. Ltd., Tokyo, Japan) 7, 5, 3 and 1 days prior, and 1, 3, 5 and 7 days after the intravenous inoculation of 10^6 conidia of an *O. gallopava* isolate (IFM 52604) per 20 g of body weight. Morbidity and mortality were monitored for 4 weeks. The mice were killed by cervical dislocation under ether anesthesia. Approximately 5 mm × 5 mm × 5 mm volume of the brain and spleen was removed and placed on a PDA plate, and was cultured at 25 °C for 2 weeks. The remaining brain and spleen tissues were fixed with 10% formalin, processed by a routine histopathological technique, stained using the periodic acid Schiff (PAS) hematoxylin method and observed under light microscopy.

DNAs from the brain and spleen were obtained from fixed tissues with 10% formalin. A piece of organ sized 5 mm × 5 mm × 5 mm was washed with distilled water and the DNA was extracted using a kit (DEXPAT®). The DNA samples were processed for nested PCR using NL-1 and NL-4 (Kurtzman and Robnett, 1997) for the first round of PCR, and OgF3 and OgB3 for the second one. The DNAs were directly processed for the LAMP method by prolongation of the incubation period for 120 min. A spleen sample from an intact mouse treated with the same volume of corticosteroid was used as a negative control for both PCR and LAMP. The present work complied in full with all relevant guidelines and policies of the Animal Welfare Committee of the Faculty of Medicine of Chiba University, Japan.

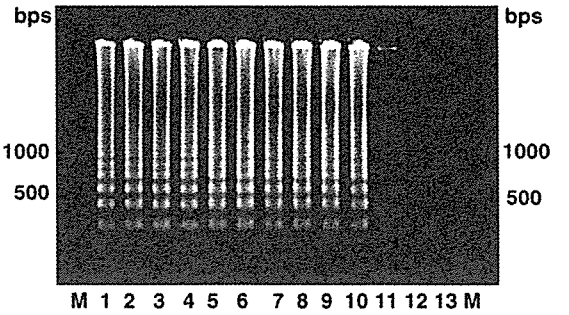

Fig. 1. DNAs extracted from *O. gallopava* isolates processed by the LAMP method. M, Marker; lanes 1–10, *O. gallopava*; 11, *O. humicola*; 12, *O. gamsii*; and 13, *O. constricta*.

3. Results

Sequence lengths, accession numbers and identity to *O. gallopava* and related species are shown in Table 1. All sequences of the D1/D2 LSU rDNA for *O. gallopava* consisting of 615 base pairs were identical except for the 421st and the 435th bases, which showed heteromers of cytosine and thymine.

The *O. gallopava* species-specific PCR primer set amplified the 375 base-pair-sized fragments only for *O. gallopava* isolates. The primer set did not react with related dematiaceous fungal species or other medically important ones. The detection limit of the species-specific PCR was 100 fg by serial dilutions of DNA (data not shown).

DNAs extracted from *O. gallopava* isolates appeared as a uniformed ladder-like band pattern by the LAMP method, while the other fungal species did not produce any bands (Fig. 1). The detection limit of the LAMP was 100 fg by serial dilutions of DNA (data not shown).

No mouse showed any behavioral change during the observation period. The fungus was found to have sprouted from all five brains and three out of five

Table 2
Pathogenicity and detection of the species-specific gene of *O. gallopava* isolate IFM 52604 from tissue samples in experimentally infected mice

Animal	Spleen				Brain			
Number	Culture	Histopathology	Nested PCR	LAMP	Culture	Histopathology	Nested PCR	LAMP
1	–	–	–	–	+	+	–	+
2	+	–	–	+	+	+	–	+
3	+	–	–	+	+	+	–	+
4	–	–	–	+	+	+	–	+
5	+	–	–	+	+	–	–	+

spleens within the incubation time. Fungal elements stained with PAS were detected in four out of five brains, and no spleens stained with PAS under light microscopy. Nested PCR was unable to detect any bands, while the uniformed ladder-like band pattern shown by the LAMP method appeared in all infected brain samples, and in four out of five samples of the spleen (Table 2).

4. Discussion

O. gallopava was found to be an independent species based on the D1/D2 LSU rDNA sequences within 99.7% of identity in the phylogenic status proposed by Kurtzman and Robnett, 1997 although its taxonomic position is still controversial. The species-specific PCR primer set and that for use in the LAMP method reacted successfully with *O. gallopava* DNA in the present experiments. Differentiation of *O. gallopava* from its related species by mycological studies take at least 4 weeks, while the species-specific PCR and LAMP methods each required under 6 h. These methods will therefore be useful for the identification and differentiation of dematiaceous fungal isolates producing two-celled conidia.

The LAMP method was recently shown to be useful in detecting SARS (Hong et al., 2004) and Newcastle disease (Pham et al., 2005). In the present study, the application of the species-specific LAMP method on the experimental *O. gallopava* infection indicated that this method is able to successfully differentiate the disease from other zoonotic diseases caused by protozoa, fungi, bacteria and viruses affecting the central nervous system and respiratory tract. In conclusion, the combinations of the LAMP method for *O. gallopava* infection, SARS and Newcastle disease will be powerful tools for the management of avian endemics.

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