表1　エキノコックス感染犬の届け出基準

1. 病原体の検出
虫体またはその一部（片節）の確認
2. 病原体の遺伝子の検出※
PCR 法による遺伝子の検出
3. 病原体の抗原の検出
ELISA 法による成虫由来抗原の検出
（駆虫治療の結果、成虫由来抗原が不検出になったものに限る）

※虫卵はテニア科条虫では形態上区別できないので遺伝子の検出を試みる。

記調査期間で3例，2005年1月の届出制施行後の1例，1997年酪農学園大学で報告された1例の計5例が確認されており，これら感染機会の少ない室内飼育犬の感染例は飼い犬への感染圧の高まりを示している。同時に，室内飼育犬は人との接触がより密接なため，人への感染源としての危険度が高いことは言うまでもない。ただし，室外飼育犬の感染例も放し飼いと密接に関連しており，犬の感染は飼育場所ではなくあくまでもネズミを食べる機会に依存することを理解してほしい。また，15頭の感染確定例のうち6頭は札幌市など市部から検出されており，都市部においても感染例があることも認識する必要がある。1997年以降の飼い犬の感染については，上記以外に，2000年の有珠山噴火時に避難住民が放逐した犬から2頭の抗原陽性例が，北海道行政がキツネの剖検調査（感染率の定点観測）時に同時に行っている犬の剖検（毎年10～20頭程度；主に捕獲犬）で1997年に1頭の感染例が報告されている。

上記調査では本州からも感染犬が検出されており，虫卵のDNA検査で1頭の感染が確定している。この犬は北海道から移動したもので，北海道で感染したものと思われる。さらに，2003～4年に国立感染症研究所が行った，フェリーによって北海道から移動した犬の調査では，検査希望者の飼い犬69頭中2頭で抗原陽性例が報告されている。犬以外では青森県の豚で感染例が報告されているが，野生動物からは報告がなく，本州にエキノコックスが定着している証拠は今のところ得られていない。

2．飼い犬のエキノコックス診断と届け出基準

エキノコックス感染犬の届け出制に対して厚生労働省が作成したガイドライン（http://www.mhlw.go.jp/topics/2004/10/tp1001-4.html）では3つの届け出基準を設けている（表1）。第一は病原体の検出で，虫体または片節を確認した場合，届け出の対象となる。虫卵の検出は，エキノコックスと他のテニア科条虫（猫条虫，胞状条虫や豆状条虫など）の虫卵が形態的に区別できないため，虫卵の検出のみでは届け出対象とならない（遺伝子診断が必要）。第二は遺伝子の検出であるが，現在エキノコックスの遺伝子検査をルーチンで行っている検査機関はなく，研究あるいは公共サービスの一環として専門機関が請け負っているのが現状である。したがって，遺伝子検査は，虫体や虫卵が検出されるなどエキノコックス感染が強く疑われる場合にかぎり確定診断のために行われている。第三は抗原の検出で，エキノコックス成虫の排泄分泌物が犬の糞便中に出てくるものを捉える方法である。本法は虫卵排出前（エキノコックスは幼虫が犬に感染してから虫卵を排出するまで約1か月を要する）の検査でも感染を検出できるため有用性の高い診断法であるが，一定の確率で偽陽性反応が検出される。北海道大学が開発した抗原検出法（環境動物フォーラムにより実施）では，0.2～0.5％の確率で偽陽性反応が検出されている。ところが，飼い犬の感染率も0.4～0.7%（偽陽性率のほぼ2倍程度）と低いため，抗原検査における陽性反応のほぼ2/3は真陽性（感染），1/3は偽陽性（非感染）となる。したがって，抗原の検出のみ（虫卵陰性）では確定診断することはできず，駆虫治療の結果、抗原が不検出になったものに限って届け出義務が発生することになる。

おわりに

北海道の登録犬は約23万頭であり，未登録犬を含めると推定30～50万頭の犬が北海道で飼育されている。したがって，相当数の飼い犬がエキノコックスに感染していると考えられ，その中には北海道外へ移動する犬も含まれるであろう。北海道で人の感染リスクを減らすために，さらには本州への拡散を防止するために，飼い主，獣医師および行政がこれらの状況を十分に認識して、ペットの適切な飼育管理と感染予防にあたらなければならない。

■

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気をつけようエキノコックス

【プロフィール】
北海道大学大学院獣医学研究科寄生虫学教室助教授。1952年、大阪生まれ。帯広畜産大学卒業後、北大大学院獣医学研究科進学。寄生虫学教室助手を経て90年より助教授。家畜や野生動物の寄生虫について研究。特に、エキノコックスについてはペットの検査法開発と野外のキツネの駆虫による感染源対策に取り組んでいる。

文／奥祐三郎
text by Yusaburo Oku

エキノコックスとは

エキノコックスは細菌やウィルスとは異なり、肉眼でも見える寄生虫の一種である。北海道に分布するエキノコックスは、和名で「多包条虫」、幼虫については特に「多包虫」と呼ばれている。主に野生動物間（キツネ・野ネズミ間）で伝播している寄生虫であるが、人にも偶発的に感染する人獣共通感染症で、北海道の重要な疾病の一つである。一般に人の寄生虫感染では一つの虫卵はそのまま一つの寄生虫に発育し、病原性も弱いのが普通である。しかし、多包虫は一つの虫卵に感染しても、その宿主（寄生される動物）の肝臓で小さな袋状になり、癌のように増殖・浸潤・転移し、強い病原性を発揮する。

現在、わが国では本症の流行は北海道に限局していると考えられているが、世界的には北半球の温帯からツンドラ地帯に広く分布し、北米やヨーロッパなどでも問題となっている。

流行状況

国内では様々な人の寄生虫病が根絶もしくは症例が激減してきたが、多包条虫症の状況は全く異なる。すなわち、一九六〇年代には北海道の一部の地域のみの風土病であったものが、一九八〇年代には全道に広がっていることが確認され、さらに、一九九〇年代には主な宿主であるキツネの感染率が上昇し、近年では約四〇％になっている。過去五年間の北海道での年間平均患者発生数は十六名と報告されているが（103ページの棒グラフ参照）、今後道外への分布拡大や患者数の増加が危惧されている。

多包条虫の一生

多包条虫の一生には、成虫の寄生する宿主（終宿主）と、幼虫の寄生する宿主（中間宿主）の二つの宿主動物が必要である。寄生虫はそれぞれ寄生する動物種（宿主域）がほぼ限定され、感染後に寄生虫が正常に発育できる好適な宿主動物だけでなく、感染はするが寄生虫の発育が抑えられる宿主動物もある。

多包条虫にとって好適な終宿主はキツネや犬などの犬科動物であり、成虫は多数の虫卵を排泄する。猫はやや抵抗性で、感染はするが成虫が虫卵を産生する例はまれである。

好適な中間宿主としては様々な野ネズミ（北海道では主にエゾヤチネズミ）が知られ、これらのネズミでは幼虫（多包虫）が活発に増殖・発育し、多数の原頭節（成虫の前段階）を産生する。一方、人や豚・馬はあまり好適な中間宿主ではない。例えば、豚や馬では肝臓に小さな限局した病変を作るのみで、原頭節も全く形成されない。人では多包虫の発育はゆっくりで、病変の中心部がしばしば壊死もしくは膿瘍になり、原頭節の産生も少ない。

多包条虫の一生は図に示したように、虫卵が好適な中間宿主（北海道では主にエゾヤチネズミ）によって食べられると、その肝臓で無数の小さな袋状の虫体となって無性増殖し、感染後一～二カ月するとその中に無数の原頭節を産生する。このような野ネズミを犬やキツネが捕食すると、小腸粘膜に原頭節が吸着し、数個の片節（卵を保有した袋）のある小型の成虫へと発育する。なお、小腸において一つの原頭節は一つの成虫となり、増殖することはない。成虫の最終片節内には虫卵が作られ、感染後一カ月すると最終片節の脱落と共に、虫卵が外界へ拡散する。残った虫体では新たな片節が再生され、この脱落・再生が繰り返されるが、犬での成虫の寄生期間は二～三カ月が多いと考えられる。虫卵はしばらく外界で生存し、低温で湿った状態では一年以上生存することもある。人は偶然にこの虫卵を摂取して感染する。

ペットへの感染の機会

キツネだけでなく、犬・猫への感染経路も野ネズミを食べたときのみである。野ネズミは自然の豊かな環境、例えば野山、自然公園、防風林、牧草地、笹藪、雑草の生い茂る荒れ地や河川敷などに生息する。このような場所で犬をリード（引き綱）から放すと、野ネズミを捕食する可能性がある。さらに、ネズミが死んだ場合でも原頭節は数日間生存しているので、道端の野ネズミの死体を拾い食いしても感染する可能性がある。キツネが時折見かけられるような郊外や農村において、屋外での放し飼いもしくはしばしばリードから放す犬では感染機会が多いと予想される。しかし、通常は室内飼いで、散歩時や郊外に連れて行った時のみにリードから放す犬でも感染例が知られている。飼い犬がネズミを食べているところを見たため犬の多包条虫検査を依

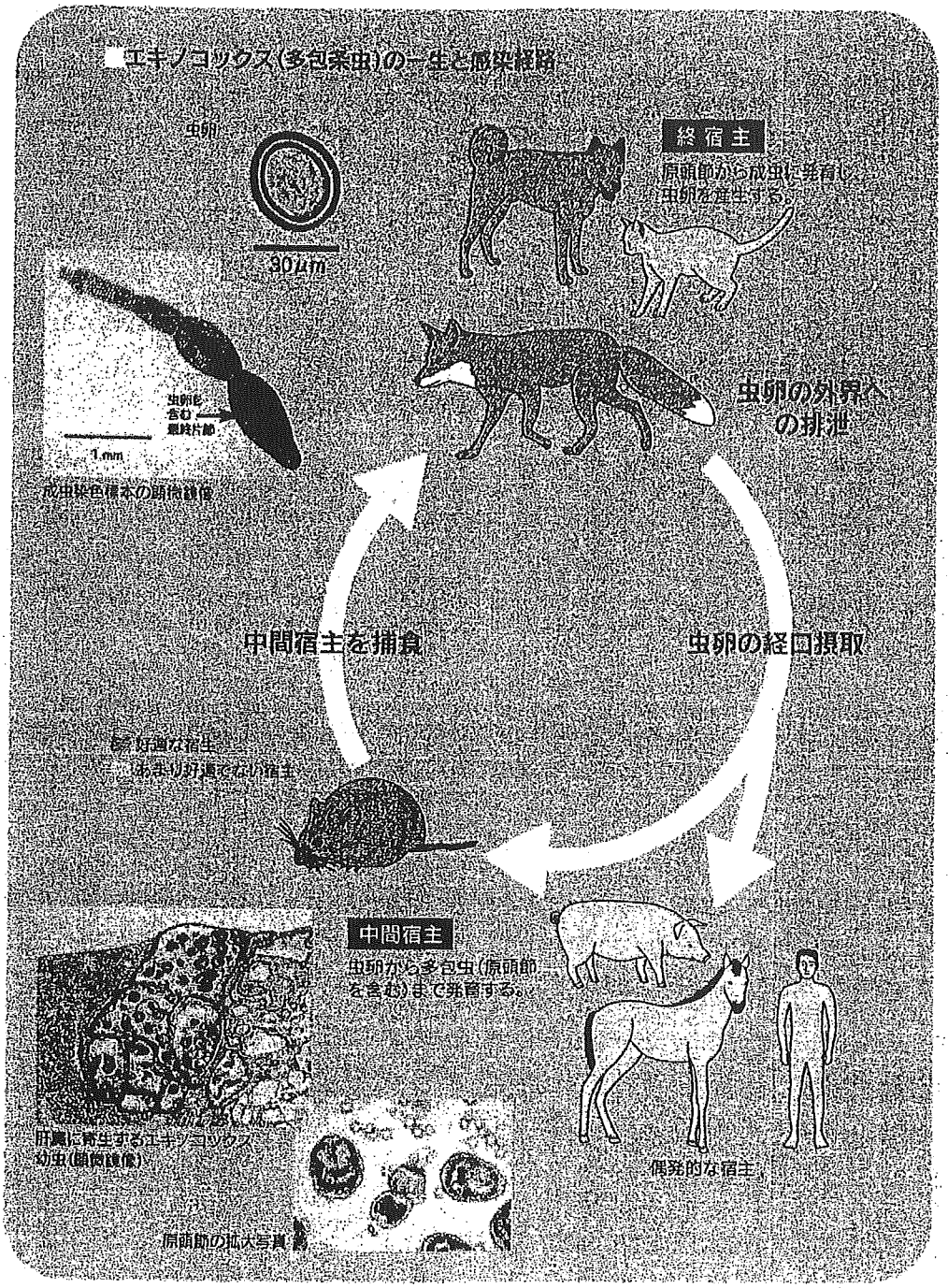
頼し、感染犬が発見された症例があるが、感染した犬の飼い主でもその犬が野ネズミを食べるとは全く予想していなかった例もある。ちなみに、現在コンパニオンとして飼育されている小型犬の品種でも、ネズミ捕獲が初期の用途であった品種もある。飼い犬がネズミを食べているところを見たかどうかだけでなく、その周辺が野ネズミの生息しそうな環境かどうか判断する必要がある。なお、キツネの感染状況は都市周辺部でも高いので、都市周辺の野ネズミも犬や猫の感染源となりうる。

感染ギツネの生息する地域でも、野ネズミの感染率は一般に低く、同一地域内でも場所による極端なバラツキがある。したがって、野ネズミを捕食した場合でも、感染ネズミを捕食することはまれと考えられる。キツネは日常的に多数のネズミを捕食するため感染率が高いが、犬はまれにのみネズミを食べるので感染率が低いものと考えられる。北海道庁による野犬・畜犬の調査を集計すると一%程度の感染率であったが、ペットの多包条虫検査を行っている環境動物フォーラムによる飼い犬の検査結果の集計では、多包条虫の虫卵を排泄していたことが確認された例は〇・三%（11／3700）であった。

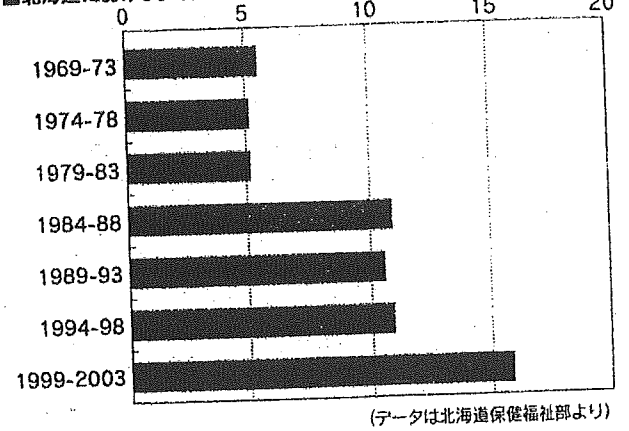
ペットを全く室外に出さない完全な室内飼いの場合は、多包条虫に感染する機会はないと考えられる。ただし、同居の猫が野ネズミを屋内に運ぶ習慣がある場合は例外である。都市部の整地され芝生のみの公園には野ネズミは生息しない。また、市街地の住家性ネズミ（ドブネズミ、クマネズミ、ハツカネズミ）がエキノコックスに感染している可能性も極めて低いので、市街地のみの散歩では犬の感染の機会はないと考えられる。飼い犬でも他の犬の糞便を食べることがあるが、もし感染した犬やキツネの糞便を食べたり、キツネの糞便のある沢水を飲んだとしても犬や猫には感染しない。

ペットが感染すると

犬や猫が感染した時には小型の成虫が小腸粘膜に吸着するだけで、ほとんど症状を示さない。しかし、北海道の飼い犬の症例で下痢を示し、その下痢便中に成虫が検出された例があることから、重度に感染した場合は下痢を引き起こすこともあると考えられる。なお、感染ネズミでは数十万以上の原頭節を保有していることもあるので、一匹のネズミの捕食でも重度感染することがある。感染犬は虫卵を糞便と共に排泄するので、人の感染



■北海道における多包虫症の年間発生患者数(各5年間平均)の推移



(データは北海道保健福祉部より)

■ペットの管理

1 ペットが感染する機会は野ネズミの捕食もしくは死体の拾い食いのみ！　これを防げば感染しない！（犬や猫は虫卵を食べても感染しない。したがって、感染ギツネや感染犬との直接的な接触や、それらの糞便を食べても感染しない。キツネの糞便が混入した沢水を日常的に飲んでも感染しない。さらに、感染した豚や馬の生の肝臓を食べても、原頭節がないので感染しない）

2 野ネズミの生息するような場所で犬のリードを放した場合は、感染の可能性がある。感染の機会が多い場合は駆虫もしくは検査が必要である。

源となる可能性があり、感染犬は無症状で飼い主が感染について気づかないので、野ネズミ捕食の機会の有無について十分気をつける必要がある。なお、感染した犬やキツネは自分が糞便と共に出した虫卵を偶発的に摂取することも多いと考えられるが、幼虫が肝臓に寄生した例は極めてまれであり、虫卵感染に対しては抵抗性である。

猫については放し飼いが多く、ネズミを捕食することも多いので、感染の機会は犬より多く、北海道庁の剖検調査では九十九頭中五頭から多包条虫の未熟成虫が発見されたが、虫卵のある成虫は含まれていなかった。ヨーロッパにおいても猫はやや抵抗性の動物であるが、時折虫卵を排泄することが知られている。

ペットの診断・治療について

犬や猫のエキノコックス診断法としては遺伝子（ＰＣＲ）診断、虫体の同定、虫卵検査、糞便内抗原検査などがあり、遺伝子診断や虫体の同定は研究機関や大学に依頼することとなるが、糞便内抗原検査はスクリーニング法として有用で、飼い主は獣医師を介して糞便を送付して検査を依頼できる。この方法で陽性の場合には、確認のための検査をさらに行うこととなる。もし感染が確認された場合は駆虫薬を投与して駆虫するが、同時に虫卵対策のために糞便の適切な処置が重要であり、獣医師や保健所の職員のサポートが必要となる（プライバシーについては守られる）。なお、感染症法の改正により二〇〇四年十月から獣医師がエキノコックス感染犬を発見した時には所轄の保健所に直ちに届け出ることが義務づけられている。

犬の多包条虫に対する駆虫薬としてはプラジカンテルが非常に効果的で、安全域もかなり広い。

虫卵は乾燥や熱に弱く、日光にも長時間さらせば死ぬ。一般の消毒剤では虫卵は死なないが、高濃度のブリーチは効果的である。

飼い主と地域における対策について

以上のことから、ペットの飼い主は飼い犬が多包条虫に罹らないように、すなわち野ネズミを食べないように注意しなければならない。また、日常的に犬の糞便の適切な処理に心がけなければならない。犬が感染した場合でも感染後に虫卵が排泄されるまでの期間に駆虫薬を投与することによって、虫卵の排泄を阻止することができる。犬に感染の機会がありそうな場合は、安全および確認のために駆虫および検査が勧められる。猫はやや抵抗性の宿主であるが、郊外や農村部で野ネズミをよく捕まえる猫については、検査もしくは駆虫も考えるべきと思われる。

犬や猫の感染予防としては、野ネズミを食べさせないことが重要であるが、この野ネズミへの感染源であり、最も重要な自然界の終宿主であるキツネに対する対策が、地域レベルの対策としては肝心である。私たちの研究も含めてキツネへの駆虫薬入りベイト（餌）の配布による感染源対策が試みられ、成果がいくつか報告されている。北海道の豊かな自然を満喫するために、自然公園へも多くの住民やペットが訪れるが、犬を放しても安全な公園が望ましい。このような限定された地域での感染源対策は可能と考えられる。

人の早期診断の重要性

北海道の現状ではキツネの感染率が高く、人の生活環境が多包条虫の虫卵に汚染され、人が感染する可能性がある。しかし、人ではエキノコックスの病気の進行はゆっくりで、早期発見により完治率が極めて高くなるので早期診断が重要である。肝臓の超音波や画像診断でも多包条虫の病変が検出できるが、北海道では自治体により検診（血清診断）が行われているので、道民は受診することが望ましい。全道的にキツネの感染率が上昇しているのにもかかわらず、一九九〇年度の受診者数約九万から、二〇〇三年度には約五万と年々減少しているが、多包条虫を侮ってはならない。

まとめ

以上のように、通常ペットの多包条虫の感染の機会は少なく、虫卵を排泄しているペットは少ないので、むやみに心配する必要はないが、ペットと人との関係は密接であるので、侮らず、ペットの適切な管理・駆虫および検査により、人とのより安全な関係が保たれるように飼い主は心がける必要がある。

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EMERGING INFECTIOUS DISEASES

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Conference Summary

Symposium on Infectious Diseases of Animals and Quarantine

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The Japan–Taiwan Symposium on Infectious Diseases of Animals and Quarantine, sponsored by the Japan Interchange Association, was held October 20–21, 2004, at the Sapporo Convention Center. The symposium focused mainly on human health and food safety. Issues that were discussed included 1) infectious diseases of animals that caused economic loss, and those diseases that threaten the health of companion animals, 2) infectious diseases that are transmitted from animals to humans, 3) emerging infectious diseases that have been reported recently in world news, such as bovine spongiform encephalomyopathy and avian influenza, and 4) animal quarantine measures to prevent the spread of the aforementioned diseases. Challenges posed by infectious diseases of animals that were faced in the past, are being faced now, and will be faced in the future were highlighted in the 3 plenary lectures.

The first plenary lecture described how Japan struggled to control rabies, Japanese encephalitis, Korean hemorrhagic fever, anthrax, and other zoonoses after the Second World War (1941–1945) when poverty and poor sanitary conditions were common in Japanese. The second plenary lecture reported on the economic loss to the pig industry in Taiwan brought about by diseases such as foot and mouth disease and circovirus infection. Also described were the efforts of Taiwanese public health officials to control the epizootic diseases through the strategic use of vaccine.The third plenary lecture described our responses to a variety of risks and focused on the necessity of risk management by an ideologically mature society when it is challenged. The need to integrate diverse expertise was reiterated and

examples of collaboration with mass media to solve problems were presented.

Speakers reported on the recent outbreaks of avian influenza in Yamaguchi, Oita, and Kyoto; the crisis control measures being implemented to curb the spread of the disease; and Dr. S. Y. Lin, Bureau of Animal and Plant Health Inspection and Quarantine, Taiwan, and finding and eradicating the H5N2 subtype of avian influenza virus in Taiwan. The pathogenic H5N1 avian influenza virus was found in 6 ducks in the Taiwan Sea straits. The ducks had been thrown overboard by smugglers on a boat from mainland China who were being pursued by the coast guards at Kinmen Prefecture. This occurrence illustrates that infectious diseases know no national boundary and underscores the need for a global surveillance system to prevent the spread of the virus.

One session detailed how an echinococcosis transmission was interrupted and monitored. Praziquantel-laced bait was distributed to wild foxes in Hokkaido in a mass deworming program attempting to interrupt the transmission cycle; the efficacy of the program was monitored by using the worm coporantigen detection method. This method did not disrupt the movement or eco-hierarchy of the red fox population and was sustainable in its efficacy. The drug-laced bait was produced by using marine waste products. Prevalence of echinococcosis in the red foxes in Hokkaido was reduced by using this method (1,2). This method was also successful in reducing the prevalence of the cestode infection in Zurich, Switzerland (3). Knowledge of being protected against infection will instill a sense of safety among the inhabitants, which in turn will lead to increased agricultural activities, as well as developing tourism industry in the region.

The control policy against echinococcosis was presented, as was the manditory reporting of infection in dogs, which became part of a revised law that became effective after October 1, 2004. This implementation is the first of its kind in the world.

Also noted was that a mutual understanding and consensus must exist among all parties concerned to effectively eradicate *Echinococcus* from red foxes. The stakeholders in this case are the inhabitants of the area, government officials, researchers, farmers, and tourist organizations.

Contemporary society is confronted with many forms of risk, including infectious disease risks and risks that threaten our food safety, that need urgent attention. This symposium brought researchers, government officials, and members of the public from Taiwan and Japan together to facilitate the bilateral regional exchange of information, with the understanding that technology that is applicable to both regions can also be extrapolated to the whole world. With the current pace of globalization, the concepts demonstrated in this symposium can serve as worldwide models.

After the symposium, the Taiwan delegation was asked for a comment on future development. The answer was that the symposium started on a bilateral basis but the discussion should be expanded to include other neighboring countries, such as Korea. Too strong a sense of nationalism may not protect national interests or the wellbeing of a nation itself.

Regarding the control of infectious diseases in animals, the symposium urged that research and development efforts tailored to specific needs be conducted.

Government authorities should not ignore problems and leave them for future generations to solve. Professional organizations, such as veterinary associations, should address the problems directly; scientific societies should not limit their activities to meetings or congresses. When these aspirations take shape, the world will have changed for the better.

The abstract of this symposium is published in Japanese and English and can be obtained through a request to Kamiya Masao.

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nematodeESTs/), we found cDNA clusters putatively encoding cytosolic Cu/Zn-SODs (cluster BMC00677). When aligned with these sequences, our sequence exhibited high sequence identity that ranged from 97 to 98%. The gene cloned in this study might represent the Cu/Zn-SOD of *B. malayi*. Because we could not find any evidence that cytosolic Cu/Zn-SOD of *B. malayi* is membrane bound, it might not play a major role in protecting parasites from oxygen-mediated killing mechanisms of the hosts; instead, it might be involved in the survival of parasites by detoxifying intracellular superoxide radicals generated by cellular metabolism.

Overexpression of enzymatically active recombinant *B. malayi* Cu/Zn-SOD would help us to investigate the enzymatic characteristics and molecular structure of the enzyme in detail. The pathophysiological and biological role of the enzyme in host–parasite interaction should also be further elucidated.

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Modified Sugar Centrifugal Flotation Technique for Recovering *Echinococcus multilocularis* Eggs From Soil

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ABSTRACT: Among soil-transmitted parasitic diseases, alveolar hydatidosis due to the ingestion of *Echinococcus multilocularis* eggs is becoming a serious problem in Hokkaido, the northern most island of Japan. Dissemination of the infection far from the endemic areas can occur if motor vehicles transmit soil contaminated with eggs. No appropriate and validated method for recovering the taeniid eggs from soil is available. A modified sugar centrifugal flotation technique, using a sucrose solution of specific gravity 1.27 and 0.05% Tween-80, was evaluated as a method to successfully recover eggs from soil. Contamination levels as low as 10 eggs per gram could be detected. This method may be useful to determine the prevalence of *E. multilocularis*, its transmission, and the potential for by monitoring soil contamination with eggs.

Soil is contaminated with various kinds of helminth eggs, which can be transmitted to humans and other animals (Mizgajska, 1997; Chongsuvivatwong et al., 1999). *Echinococcus multilocularis* is the causative agent of alveolar hydatidosis and is distributed widely in the Northern Hemisphere. *Echinococcus multilocularis* eggs are deposited in the environment by defecation from the infected definitive hosts, mainly foxes. Human infection with *E. multilocularis* is more likely to occur by accidental ingestion of eggs in soil, contaminated vegetables, water etc., rather than through direct contact with definitive hosts, except dogs. Various techniques have been developed for ascarid egg detection from the soil (World Health Organization Chronicle, 1968; Uga et al., 1989; Ruiz de Ybanez et al., 2000). However, few reports for recovering taeniid eggs from soil are available.

A method for recovering ascarid eggs from sand (Uga et al., 1989) was adapted to a sugar centrifugal flotation technique (Ito, 1980) using a sucrose solution of specific gravity 1.27 (Nonaka et al., 1998). Soil was collected from the ground of Hirosaki University School of Medicine located in a nonendemic area for *E. multilocularis*. Twenty grams of soil mixed with 20 (equivalent to 1 egg per gram [EPG]), 200 (10 EPG), or 2,000 (100 EPG) *E. multilocularis* eggs was placed in 50-ml conical tube to which was added 40 ml of 0.05% Tween-80. The eggs were not infective, having been preserved in 70% ethanol since 1969.

TABLE I. Modified sugar centrifugal flotation technique for recovering *Echinococcus multilocularis* eggs from 20 g of soil.

Time of incubation	Number of eggs detected (mean ± SD)†		
	20‡ (1 EPG)	200‡ (10 EPG)	2,000‡ (100 EPG)
2 hr	0.3 ± 0.5 (3/10)§	1.5 ± 0.7 (10/10)**	64 ± 18.7 (10/10)***
Overnight	0.2 ± 0.4 (2/10)	8.1 ± 3.7 (10/10)***	137 ± 40.8 (10/10)***
Total	0.5 ± 0.5 (5/10)	9.6 ± 4.0 (10/10)***	201 ± 54.5 (10/10)***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
† Number of detected eggs were evaluated by Student's *t*-test and found significant, where $P < 0.05$, compared with that of sample containing 1 EPG.
‡ Number of eggs added.
§ Number of tubes detected eggs/examined (n = 10).

The mixture was stirred vigorously and sieved through 100-μm mesh. The suspension was centrifuged at 1,000 *g* for 5 min, and the supernatant was discarded. The sediment was resuspended in 7–8 ml sucrose solution (1.27 specific gravity). The suspension was transferred to 15-ml tubes and centrifuged again at 1,000 *g* for 15 min. Tubes were filled to the top, and a coverslip (24 × 24 mm) was placed on the tube. Coverslips were examined microscopically 2 hr later. A new coverslip was placed on the top of tube and left overnight to detect the remaining eggs. Statistical significance of the results was determined using Student's *t*-test. Data were expressed as mean ± SD, and a *P* value of less than 0.05 was taken as the minimum level of significance.

Eggs were detected in all soil samples added with 100 EPG after 2 hr of incubation. More eggs were detected after overnight incubation compared with 2 hr of incubation. As shown in Table I, the egg recovery rate was 2.5, 4.8, and 10% in soil samples containing 1, 10, and 100 EPG, respectively. The egg detection rate significantly increased with increase in EPG. The present method could detect contamination of soil with taeniid eggs at a concentration of 10 EPG.

An average of 300 eggs per gravid segment was counted in *E. multilocularis* collected from the fox (Zeyhle and Bosch, 1982). Therefore, if only 1 gravid segment was mixed with 20 g of soil, the EPG in this locality will be more than 10. A total of 34,000 *E. multilocularis* adults were recorded from a heavily infected fox in endemic area of Japan (Morishima et al., 1999). *Echinococcus multilocularis* eggs deposited in feces and dispersed into soil could clearly contaminate a wide area. Furthermore, if fox heavily infected with *E. multilocularis* was involved in a motor accident, a huge number of eggs could be disseminated into the environment from injured viscera. According to Tsukada et al. (2000), infected foxes frequently encountered traffic accidents in Hokkaido, Japan. Therefore, motorcars contaminated with eggs could disperse the parasite to nonendemic areas.

It is somewhat difficult to evaluate the sensitivity of present techniques for application to field survey. However, the contamination of soil with *E. multilocularis* is not the same in different areas. The contamination of eggs in soil should be more intense around fox dens, showing that the wild rodents, its intermediate host, captured near the dens were heavily infected (Kamiya et al., 1977). Therefore, the sensitivity level of 1 EPG is still valuable for detection of eggs in certain instances such as in the soil of highly endemic areas or soil adhered to motorcars run over the injured fox viscera infected with *E. multilocularis*.

Recently, the present technique adapted for 2 kg of soil was used in a survey for detecting *E. multilocularis* eggs in soil left in the Hokkaido to Aomori ferryboat to monitor the transmission of eggs from endemic to nonendemic area. Although no helminth egg was detected, *Isospora* oocysts, mites, and eggs of mites were found (Matsuo et al., 2003). Of course, the soil collected from the endemic area must be incubated at 70 C for 12 hr (Nonaka et al., 1998) or frozen at −80 C for 3 days or more (Veit et al., 1995) to inactivate egg infectivity.

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Laboratory and Epidemiology Communications

A Coprological Survey of the Potential Definitive Hosts of *Echinococcus multilocularis* in Aomori Prefecture

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Alveolar echinococcosis (AE) is a zoonotic helminth disease classified as Category IV by the Infectious Diseases Control Law of Japan, which requires notification of both human and canine cases of the disease. The known domestic distribution of the causal agent, *Echinococcus multilocularis*, is exclusively restricted within Hokkaido; thus, human AE infections occur predominantly in the inhabitants of this region. On the other hand, there have also been AE patients reported from prefectures other than Hokkaido. Although many of these cases are thought to have had contact with either Hokkaido or foreign endemic countries, some infections were probably acquired autochthonously. It is noteworthy that half of the autochthonous cases were recorded in Aomori Prefecture (1), which is situated on the northern tip of the mainland of Japan and faces Hokkaido across the Tsugaru Strait (Fig. 1). Furthermore, slaughter pigs infected with metacestodes of *E. multilocularis* have recently been reported in this area (2). These data imply the spread of the parasite from Hokkaido to Aomori Prefecture, but adult *E. multilocularis* has not yet been detected in necropsy surveys on wild red foxes, though it must be kept in mind that relatively few animals were studied (3,4).

Necropsy diagnosis of *Echinococcus* in the small intestines of the definitive hosts is laborious, time-consuming and even biohazardous when it is performed on a fresh carcass, but nevertheless serves as a gold standard owing to its reliability. However, postmortem examination is not suitable for areas like Aomori Prefecture, where animal carcasses are sparse or unavailable. According to game bag records, for instance, the mean annual number of red foxes captured in this prefecture during 1999-2001 was less than 100 (5) (Full data are available at http://www.sizenken.biodic.go.jp/wildbird/flash/toukei/07toukei.html). Although domestic dogs can also become the definitive hosts of the parasite, they cannot be included in necropsy surveys for ethical reasons. Therefore, we attempted a survey with antemortem diagnostic approaches to increase the target population.

The survey was undertaken on potential definitive hosts in Aomori Prefecture from December 2003 to February 2005. Forty-three fecal samples of wild red foxes, which included 17 rectal feces of shot individuals and 26 naturally excreted feces in fields, were collected by local hunters. With regard to the collection of the latter samples, local hunters were instructed in the criteria of vulpine feces (6) in order to avoid sample contamination with other carnivores. In addition, 134 fecal samples of hunting dogs were collected due to their management in fields in which they may acquire the infection by preying on rodent intermediate hosts. All samples were stored at −20°C until used. Microscopic egg detection was conducted with both a modified Wisconsin procedure (7) using a sucrose solution whose specific gravity was 1.27 and a formalin-ethyl acetate sedimentation technique (8). Because the eggs of *Echinococcus* morphologically resemble those of other taeniid tapeworms (9), they were subjected to PCR as described below. *Echinococcus* coproantigen was screened using an ELISA kit from Dr. Bommeli AG (CHEKIT®-Echinotest; Liebefeld-Bern, Switzerland) following manufacturer's instructions. The results of the test were expressed as percentages of likelihood for infection using the following equation: relative positivity (%) = {(OD of test sample−OD of negative control)/(OD of positive control−OD of negative control)} × 100. A relative positivity value of less than 30% was regarded as a negative and a value greater than 40% was regarded as a positive. Values between 30-40% were considered to be indeterminate. The test has a sensitivity rate of 90.9% and a specificity rate of 98.8% when applied to a group with a prevalence of 8% (data from the manufacturer). To confirm the results of the coproantigen test, and to distinguish *E. multilocularis* from other taeniid tapeworms, a specific nested PCR test (10) was carried out. The extraction of DNA from fecal samples and egg materials was performed using a commercial kit (QIAamp DNA Stool Mini Kit; Qiagen GmbH, Hilden, Germany) and the protocol described by

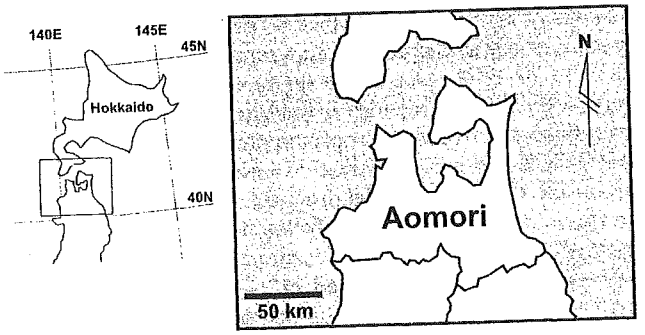

Fig. 1. Location of Aomori Prefecture, Japan.

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Bretagne et al. (11), respectively.

Taeniid eggs were detected in two vulpine and one canine fecal samples, but all were negative in the *E. multilocularis*-specific PCR test. Taking both host groups together, only a single dog gave a positive result for coproantigen. The dog was treated by oral administration of 5 mg/kg bodyweight of praziquantel (Droncit®; Bayer AG, Leverkusen, Germany), and feces excreted after the chemotherapy were taken daily until 2 days post-treatment. A follow-up coproantigen test was performed on post-treatment fecal samples. The relative positivity values yielded showed a remarkable change: the value had reduced from 44% at pretreatment to 10% at 2 days after treatment. This could be translated into success in deworming *E. multilocularis*, which is susceptible to chemotherapy with praziquantel. However, in the PCR test, no amplification products were obtained from any samples tested. The fecal samples were subjected to further parasitological examination. All remnants were slurried in an adequate volume of tap water and washed by decantation, and the sediments were scanned under a stereomicroscope at magnifications of 20-50×; no parasite segments were recovered. The reason for this discrepancy is unclear, but may be explained by a false positive of the coproantigen test or false negatives of the PCR and parasitological examination. Considering the reactivity to the taeniacidal drug, the false positive observed in the coproantigen test might have been caused by other taeniid cestodes. Such cross-reaction of the coproantigen test has previously been reported (12,13), however, no DNA fragments were amplified in the first round of the nested PCR test which detects cyclophillid cestodes, and no worm debris was found in the fecal sediments. The false negatives may relate to the degree of maturation and/or low infection intensity of *E. multilocularis*, both of which could give false negative results in the PCR test (10). Since adult *E. multilocularis* is a tiny worm, and because its proglottids will be broken off by the pharmacological action of praziquantel (14), it may be overlooked in parasitological examination. We are therefore not fully convinced of the existence of *Echinococcus* infection in this dog. Further study is needed to improve the confirmation test.

Once AE is established in an area, elimination is quite difficult. Taking into account its public health consequences, monitoring of the parasite among potential definitive hosts must be a continuous task until an effective countermeasure is established.

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rinii に対し抗体反応が陽性であった。刺咬マダニ種は *Ixodes persulcatus*（シュルツェマダニ）と同定された。また大原綜合病院における抗体調査により，野兎病，ブルセラ症，日本紅斑熱，チフス熱，およびQ熱は否定的であった。

マダニに刺咬された記憶は本人にはないが，ハバロフスクでの蝶の採集の際に現地の人からダニがいると注意されていること，蝶の採取を終えて宿へ戻った際に友人のタオルにダニがついていることを見ていることから，ハバロフスクでマダニに刺咬されたと思われた。実際シュルツェマダニはハバロフスクでは見出されるが，カンボジアでは未記載のマダニで，国内のみならずロシアでのライム病ボレリア媒介種となっている。一方で *B. valaisiana* 近縁種は韓国，中国，タイなどの東南アジアおよび本邦南西諸島で見出されているが，ハバロフスクで見出されたことはない。またこれまでシュルツェマダニから *B. valaisiana* 近縁種が見出されたことはないことから，シュルツェマダニの *B. valaisiana* 近縁種の媒介能については不明である。

これらのことから，カンボジアで *B. valaisiana* 近縁種に感染後，ハバロフスクでさらにシュルツェマダニに刺咬された可能性と，ハバロフスクでのシュルツェマダニ刺咬により *B. valaisiana* 近縁種に感染，ライム病を発症した可能性が考えられた。いずれの可能性も現時点では否定できないが，*B. valaisiana* 近縁種による感染症例は世界で初めてであり，今後 *B. valaisiana* 近縁種に対しても注意が必要であることが示された。

これまで *B. garinii* 感染が見出されているハバロフスクなどシュルツェマダニの生息地域でのマダニ刺咬には注意が必要であるとともに，*B. valaisiana* 近縁種の存在が確認もしくは推定されている，東南アジア，韓国や本邦南西諸島などでも，ライム病媒介マダニの刺咬を受けないように注意する必要がある。

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＜国内情報＞

埼玉県内の犬の糞便から検出されたエキノコックス（多包条虫）の虫卵

今般，埼玉県内で捕獲された犬の糞便から，エキノコックス（多包条虫 *Echinococcus multilocularis*）の虫卵が検出されたので，その概要について報告する。

衛生研究所および動物指導センターでは，共同研究として1999年5月から犬および猫における寄生虫類とリケッチア等の保有状況に関する調査を開始し，「埼玉県における動物由来感染症に関する実態調査」を実施している。糞便検査は2005年8月末までに，犬は550検体，猫は747検体について実施した。

動物指導センターで採材された糞便は，その当日または翌日に衛研へ搬入し，できるだけ速やかに検査を実施している。すべての検体において薄層直接塗抹法，ホルマリン・エーテル（酢酸エチル）法，ショ糖遠心浮遊法を併用し，検出された原虫類の一部に関しては，それを同定するために各種染色法や遺伝子解析を実施している。

2005年6月3日，県北で捕獲された雌犬（犬種不明）の糞便からテニア科の虫卵が検出された。犬から検出し得るテニア科の条虫には，豆状条虫（*Taenia pisiformis*），胞状条虫（*T. hydatigena*），多包条虫（*Echinococcus multilocularis*），単包条虫（*E. granulosus*）などがあるが，いずれの虫卵も形態は類似し，光学顕微鏡下での鑑別は困難である。そこで，得られた虫卵から抽出した遺伝子をPCR法で増幅し，その産物（約250bp）についてダイレクトシークエンスを行ったところ，塩基配列は既報の多包条虫（北海道由来）のものとすべて一致した。

感染症法の改正以降，国内では北海道以外の都府県からエキノコックスの虫卵が検出された初めての事例であることから，検査結果が得られた8月30日以後，直ちに関係機関による対応を開始した。

保健所などにおける住民対応を円滑に進めるために「エキノコックス症について」および「埼玉県におけるエキノコックス症についてQ & A」を作成し，生活衛生課，衛生研究所，動物指導センターのホームページに掲載した。

また，獣医師会，医師会などへの情報提供，そして保健所担当者に対する「エキノコックス症とその対策」，「県民相談に関わるQ & A」等の研修会を開催するなど，県民への十分な情報提供を行うための方策を重ねた上で，9月8日にプレスリリースを行った。

今後の対応については，飼い主，動物取扱業者に対して，北海道からの犬の持ち込みについて注意を呼びかけるとともに，飼育放棄犬で北海道との関連が確認された犬の糞便検査を実施する。また，エキノコックスが埼玉県に定着しているとは考えにくいが，今回の事例は捕獲された犬から虫卵が検出されたことから，中間宿主となる野鼠を捕獲し，エキノコックスの感染状況を調査する。

近年，エキノコックスに関しては「厚生労働省新興・再興感染症事業」（主任研究者・神谷正男）などによる研究成果が公表され，「犬のエキノコックス症対応ガイドライン 2004 － 人のエキノコックス症対策のた

めに－」が作成されるなど，その対策の重要性が再認識されている。

埼玉県では今後も動物由来感染症対策として，エキノコックス等の寄生虫に関する実態調査を継続する予定である。

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＜国内情報＞

保育園で発生した腸管出血性大腸菌O26の集団感染事例――札幌市

2005年6月29日，医療機関から保健所に男児A（3歳）について腸管出血性大腸菌（EHEC）O26を分離し，VT1(＋）の届出があり，患者および家族の健康調査から，患者は保育園に通園していることが判明し，保育園における有症者等の状況について調査および指導を開始した。

その翌週（7月5日），同医療機関から先に届出された男児Aと同じ保育園に通う男児B（2歳）についてEHEC O26, VT1(＋）の届出があったため，7月6日～8日，同保育園について患者2名を除く全園児102名，全職員26名の検便，トイレ周辺およびビニールプール（大1，小4）等のふきとり液20検体，保存食17検体のEHEC O26細菌検査を実施した。その結果，園児15名からEHEC O26が検出されたが，職員の便，ふきとり液および食品からはいずれも検出されなかった。保育園では患者の発生状況および検便検査結果から，二次感染予防のため7月11日～16日の間を自主休園とした。

翌々週（7月14日），初回検便で陰性の園児および職員の検便（2回目）を実施した結果，新たに園児2名および職員1名からEHEC O26が検出された。その後，8月9日までにさらに園児2名および陽性の園児の家族3名からEHEC O26が検出された。

8月29日，陽性者であった全員の検便の陰性が確認され，また，その後新たな発症者が見られないことから，保育園におけるEHEC O26集団感染の終息を確認した。

表1. 保育園におけるEHEC O26検査結果

種　類	検体数	陽性数	有症者	無症状保菌者
園児	104	21	14	7
職員	26	1	1	0
家族	69	3	0	3
ふきとり	20	0		
保存食	17	0		
合　計	236	25	15	10

表2. 園児クラス別陽性数

クラス	園児数	年齢(歳)	陽性数(率)	有症者	無症状保菌者
	104		21 (20%)	14	7
A	13	1	4* (29%)	4*	0
B	21	2～6	4 (19%)	2	2
C	23	2～6	7* (30%)	6*	1
D	22	2～6	3 (14%)	0	3
E	24	2～6	3 (13%)	2	1

*初発患者を含む
なお、クラスAに男児B(2歳)も在籍していた

本事例における感染者数は園児21名，職員1名および園児の家族3名，計25名であった（表1）。

保育園の園児の年齢構成は1～6歳で，13～24人のクラスが5クラスあり，1歳の1クラスと2～6歳の4クラスで，2～6歳の混合保育を主とし，1週間に2回年齢別の交流会を実施しており，陽性者は各クラスに3～7人認められた（表2）。

本事例では，有症園児で症状回復後も菌が陰性化するまでに長期間（25日）を要した例があった。また，発症後1週間の検査で菌は検出されず10日以上経過してからEHEC O26が検出された園児や，初発患者の届出から約1カ月後に発症し，EHEC O26が検出された園児の例があった。

検出された菌株のパルスフィールド・ゲル電気泳動のバンドパターンを調べた結果，検査した11株中10株のバンドパターンは完全に一致しており，1株のみが1本多かったが，類似度は97%で同一由来と判断した。

細菌検査には，従来のセフィキシム・亜テルル酸カリウム加ラムノース・マッコンキー基礎寒天培地に1%セロビオースを添加することにより，EHEC O26の選択性をさらに高めた分離培地（セロビオース加CT-RMAC）を用いた（IASR 23: 290-291, 2002参照）。また，modified *Escherichia coli* Broth（mEC）による42℃18時間増菌培養を併用した。

疫学調査および細菌検査の結果，感染源および伝播経路は特定することはできなかったが，食品などの単一曝露による感染の可能性は低く，人→人への感染の可能性が高いと考えられた。また，EHEC感染の危険因子として，幼児の衛生管理の困難さやプール遊びなどの要因が関わっていると考えられた。

過去，札幌市におけるEHEC感染症の集団発生状況は1999（平成11）年7月に1保育園でO26・VT2(＋)，2002（平成14）年7月に2保育園でO26・VT1(＋)（IASR 23: 290-291, 2002参照）であり，いずれも夏季に保育園においてEHEC O26の集団感染が発生していることから，園児の健康管理，園児・職員の手洗い等の衛生教育や夏季の簡易プールの衛生管理などの徹底が重要と考えられる。

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Identification Procedure for Pasteurella Pneumotropica in Microbiologic Monitoring of Laboratory Animals

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Abstract: *Discrepancies have been recognized in the identification of Pasteurella pneumotropica between testing laboratories. To determine the causes of the differences and to propose a reliable identification procedure for P. pneumotropica, a working group was organized and 69 isolates identified or suspected as P. pneumotropica were collected from 8 laboratories in Japan. These isolates were examined by colony morphology, Gram-staining, the slide agglutination test using two antisera (ATCC35149 and MaR), two commercially available biochemical test kits (ID test, API20NE) and two primer sets of PCR tests (Wang PCR, CIEA PCR). The 69 isolates and two reference strains were divided into 10 groups by test results. No single procedure for P. pneumotropica identification was found. Among tested isolates, large differences were not observed by colony morphology and Gram-straining except for colony colors that depended on their biotypes. Sixty-eight out of 69 isolates were positive by the slide agglutination test using two antisera except for one isolate that tested with one antiserum. The ID test identified 61 out of 69 isolates as P. pneumotropica and there was no large difference from the results of CIEA PCR. From these results, we recommend the combination of colony observation, Gram-straining, the slide agglutination tests with two antisera and biochemical test using the ID test for practical and reliable identification of this organism.*

Key words: *identification procedure, microbiologic monitoring, Pasteurella pneumotropica*

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Introduction

P. pneumotropica is a non-motile, non-hemolytic, facultative anaerobic, Gram-negative coccobacillus, which colonizes on the mucosa of the nasopharynx, trachea, lungs, vagina, uterus, urinary bladder, intestines, and other organs of laboratory animals [5, 12, 15, 18, 20–22]. The pathogenicity of this organism in immunocompetent laboratory mice and rats is known to be extremely weak although it was considered strong in the past. However, this organism can occasionally produce clinical disorders in immunodeficient animals [1, 15].

At present, *P. pneumotropica* is considered a routine test item of microbiologic monitoring of laboratory mice and rats in Japan, the US and Europe [16, 19]. However, many researchers around the world recognize that there is a serious problem concerning identification of this organism. Disagreements of test results between testing laboratories have sometimes been observed, and the same situation is also found in Japan. To minimize confusion caused by discrepancies in test results, a working group was established. The aim of the working group was to propose a new identification procedure that is both practical and reliable for this organism. Reference strains and the isolates provided by group members were checked using the same testing methods and reagents as the ICLAS Monitoring Center.

Materials and Methods

Reference strains and isolated strains

ATCC35149 (biotype Jawetz) and CNP160 (biotype Heyl) were used as reference strains. Sixty-nine isolates from 34 mice, 25 rats, 7 hamsters, 2 rabbits, and 1 guinea pig, which were identified or suspected as having *P. pneumotropica* by biochemical characteristics, were collected from 8 laboratories in Japan.

Examinations

1. Morphological examinations

All strains were plated directly onto 5% horse blood agar plates (Climedia; Sanko Junyaku Co., Tokyo), incubated aerobically at 37°C for 48 h, and examined for colony morphology. Gram-staining (Favor G; Nissui Pharmaceutical Co., Tokyo) was also performed for each isolate.

2. Immunological and biochemical examinations

The slide agglutination test was performed as an immunological examination using rabbit antisera of a mouse isolate ATCC35149 and a rat isolate MaR that were gifts from NIH Japan. Two commercially available identification kits (ID test HN 20 rapid; Nissui Pharmaceutical Co., Tokyo and API20NE; Nihon Biomeriux, Tokyo) were used for biochemical examinations.

3. DNA examination

DNA extraction

All strains were propagated in brain heart infusion broth (Difco Laboratories, MI, U.S.A.) supplemented with 5% horse serum for preparation of DNA extract. Three methods (E.N.Z.A. Bacterial DNA Kit; Omega Bio-tek, GA, U.S.A., MagExtracter Genome; TOYOBO Co., Osaka, and SDS methods) were utilized for bacterial DNA extraction. The methods were selected based on the harvested DNA volume of each strain.

PCR test

Two primer sets reported by Wang *et al.* (Wang's PCR) and Nozu *et al.* (CIEA PCR) were used for PCR tests [17, 20]. Samples(4 µl) containing 60 ng of genomic template DNA were added to 16 µl of PCR mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 mM dNTP mixture, 0.4 µM sense primer,0.4 µM antisense primer, and 5 units of Taq polymerase (Takara Shuzo Co., Kyoto). Each mixture was placed in a programmable thermal cycler (Gene Amp PCR System Model 9700; PE Biosystems, Calif, U.S.A.). PCR was performed according to Wang's or Nozu's procedure. The PCR amplification products were analyzed by gel electrophoresis in 4% agarose.

16S rDNA sequence analysis

Analysis of 16S rDNA sequencing was performed for 35 isolates from 17 mice, 13 rats, 3 hamsters, 1 rabbit, and 1 guinea pig. The five primers used for 16S rDNA sequencing and PCR are listed in Table 1. The templates were amplified by PCR using primers PAS-1 and PAS-2C. Each PCR product was sequenced by an automated sequencer (310 Genetic Analyzer; Applied Biosystems Japan, Tokyo). The data were analyzed using a software package, DNASIS (Hitachi Software Engineering, Tokyo), and compared with the sequences of *P. pneumotropica* biotype Jawetz (M75083) and Heyl (AF012090), *P. dagmatis* (M75051), *P. aerogenes* (M75048), *P. multocida* subsp *multocida* (M35018) and

Table 1. Primers for *P. pneumotropica* complete 16S rDNA sequencing

Primer[a)]	Sequence (5'–3')	Position[b)]
PPSEQ-1	ACTCCTACGGGAGGCAGCAG	331–351
PPSEQ-2C	CACATGAGCGTCAGTACAT	738–756
PAS-1	ATTGAAGAGTTTAGTCATGG	2–21
PAS-2C	TGAATCATACCGTGGTAAAC	1452–1471
16S-1	AACAGGATTAGATACCC	773–788

a) PAS-1 and PAS-2C were used for DNA amplification and sequencing. b) Positions within the *P. pneumotropica* 16S rDNA sequence (M75083) corresponding to the 5' and 3' ends of each primer.

Actinobacillus muris (AF024526) in the Gen Bank database for homology analysis [4, 6, 7, 17].

CLUSTAL W version 1.6 was used for multiple alignments of the data and the sequence of *P. pneumotropica* biotype Jawetz (M75083) and Heyl (AF012090), *P. dagmatis* (M75051) and *A. muris* (AF024526).

Results

Reference strains

The results for the two reference strains are shown in Table 2. Both strains showed smooth colonies 3–5 mm in diameter on blood agar. The colony color of biotype Jawetz was grayish-white and that of biotype Heyl was yellow. They were nearly identical morphologically, Gram-negative short rods or coccobacilli. Both of them were positive in the slide agglutination test, and identified as *P. pneumotropica* by the ID test and API20NE kits. Both strains were detected by the CIEA PCR test. However, biotype Heyl was not detected by Wang's PCR test. The 16S rDNA sequences of biotype Jawetz and Heyl determined in this study were more than 99% identical with those in the Gene Bank database. The 16S rDNA homology between the two biotype strains was under 96%.

Isolates

All 69 isolates showed nearly identical smooth colonies 3–5 mm in diameter on blood agar. Colors were grayish-white or yellow, the same as for the reference strains. Most of them had Gram-negative short rods or coccobacilli, and only three isolates from rat and hamster showed slightly longer shapes.

Thirty-three out of 34 mouse isolates, 11 out of 25 rat isolates, 5 out of 7 hamster isolates, all of 2 rabbit isolates and 1 guinea pig isolate showed agglutination with ATCC35149 antiserum. However, none of the 14 rat isolates agglutinated with ATCC35149 antiserum, but they showed agglutination with MaR antiserum. Sixty-eight of 69 isolates tested with one antiserum were positive by the agglutination test with either antiserum of ATCC35149 and MaR (Table 2). The exception was a mouse isolate.

The ID test identified 61 isolates (30 mouse, 21 rat, 7 hamster, 2 rabbit and 1 guinea pig isolates) as *P. pneumotropica*, but API20 NE identified only 39 isolates (11 mouse, 18 rat, 7 hamster, 2 rabbit and 1 guinea pig isolates) as *P. pneumotropica* (Table 3).

CIEA PCR detected 63 out of 69 isolates and all of 6 isolates which were not detected by CIEA PCR were of rat origin. Wang PCR detected only 27 isolates which consisted of 18 mouse isolates, 7 rat isolates and 2 hamster isolates (Table 3).

Thirty-four out of 35 isolates (16 mice, 13 rats, 3 hamsters, 1 guinea pig and 1 rabbit) showed over 96% homology with the *P. pneumotropica* Jawetz or Heyl 16S rDNA sequence on the database. One isolate classified as *P. pneumotropica* by the agglutination test, API20NE test and CIEA PCR showed 98% homology with *Actinobacillus muris* (Table 2).

The 16S rDNA sequences of 35 isolates, *P. pneumotropica* biotypes Jawetz and Heyl, *P. dagmatis* and *A. muris* could not be classified by cluster analysis of multiple alignments as shown in Fig. 1.

Discussion

First, we will discuss the cause of the discrepancies in identification of *P. pneumotropica* in each testing laboratory. This organism has two biotypes, Jawetz and Heyl. The sequence of 16S rDNA of these biotypes showed that they are different species. *P. pneumotropica* formerly had the Henriksen biotype, which is now classified as a different species, *Pasteurella dagmatis* [14]. Sixty-nine isolates were divided into 10 groups according to biochemical and PCR tests as shown in Table 2. This result shows that *P. pneumotropica* has characteristics too wide for one species. The same conclusion was also drawn from the sequence data of 35 isolates that could not be clearly

Table 2. Characteristics of reference strains and grouping of isolates depending on their characteristics

Group	Origin	Agg[b]		Biochemical test		PCR tests		Sequence[e]	
		ATCC	MaR	ID test	API20NE	CIEA	Wang	Jawetz	Heyl
Reference strains									
P. pneumotropica									
Jawetz (ATCC35149)		O	O	O[c]	O[d]	O	O	99%	–
Heyl (CNP 160)		O	O	O	O	O	×	–	99%
Isolates									
A	Mouse	O		O	O	O	O	99%	–
	Mouse	O	O	O	O	O	O	98%	–
	Rat	O		O	O	O	O	97%	–
	Hamster	×	O	O	O	O	O	97%	–
	Rat (4)[a]	×	O	O	O	O	O		
	Hamster	×	O	O	O	O	O		
B	Mouse (3)	O		O	×	O	O	99%	–
	Rat	O		O	×	O	O	99%	–
	Mouse	O		O	×	O	O	98%	–
	Mouse (6)	O		O	×	O	O		
	Mouse (3)	O	O	O	×	O	O		
C	Mouse	O		O	O	O	×	98%	–
	Rat	×	O	O	O	O	×	96%	–
	Hamster	O		O	O	O	×	–	99%
	Rat (2)	O		O	O	O	×	–	98%
	Rat	×	O	O	O	O	×	–	98%
	Mouse	O	O	O	O	O	×	–	97%
	Hamster	O		O	O	O	×	–	97%
	Rabbit	O		O	O	O	×	–	97%
	Mouse	O		O	O	O	×	–	96%
	Guinea pig	O		O	O	O	×	–	96%
	Mouse (5)	O		O	O	O	×		
	Rat (2)	×	O	O	O	O	×		
	Rat (3)	O		O	O	O	×		
	Hamster (3)	O		O	O	O	×		
	Rabbit	O		O	O	O	×		
D	Mouse (5)	O		O	×	O	×	98%	–
	Mouse	×		O	×	O	×	96%	–
	Rat	O		O	×	O	×	96%	–
	Mouse	O		O	×	O	×		
	Rat	×	O	O	×	O	×		
E	Rat	O		O	×	×	×	96%	–
	Rat	O		O	×	×	×		
F	Mouse	O		×	×	O	O	99%	–
	Rat	×	O	×	×	O	O	96%	–
	Mouse (2)	O		×	×	O	O		
G	Mouse	O		×	O	O	×	*Actinobacillus muris*[f]	
	Rat	×	O	×	O	O	×	96%	–
H	Rat	×	O	O	O	×	×	97%	–
	Rat	O		O	O	×	×	96%	–
I	Rat	×	O	×	O	×	×	96%	–
J	Rat	×	O	×	×	×	×		

[a] Number of strains showing same characteristics. [b] Slide agglutination test. ATCC is antiserum of ATCC35149. MaR is antiserum of MaR. O: positive, ×: negative; Blank: not tested. [c] O: %id 100, ×: %id 0. [d] O: %id 96–99.9, ×: %id 0–37. [e] 16S rDNA Sequence data were compared with six bacteria data: *P. pneumotropica* biotype Jawetz and Heyl, *P. dagmatis, P. aerogenes, P. multocida* subsp *multocida, A. muris.* –: under 96% homology. Blank: not tested. [f] This isolate showed 98% homology with *A. muris.*

Table 3. Comparison of two biochemical kits and two PCR tests for detection of *P. pneumotropica*

		Isolates from					Total
		Mouse	Rat	Hamster	Rabbit	Guinea pig	
Biochemical test	ID test	30/34[a)]	21/25	7/7	2/2	1/1	61/69
	API 20NE	11/34	18/25	7/7	2/2	1/1	39/69
PCR tests	CIEA PCR	34/34	19/25	7/7	2/2	1/1	63/69
	Wang PCR	18/34	7/25	2/7	0/2	0/1	27/69

a) Number positive / number tested.

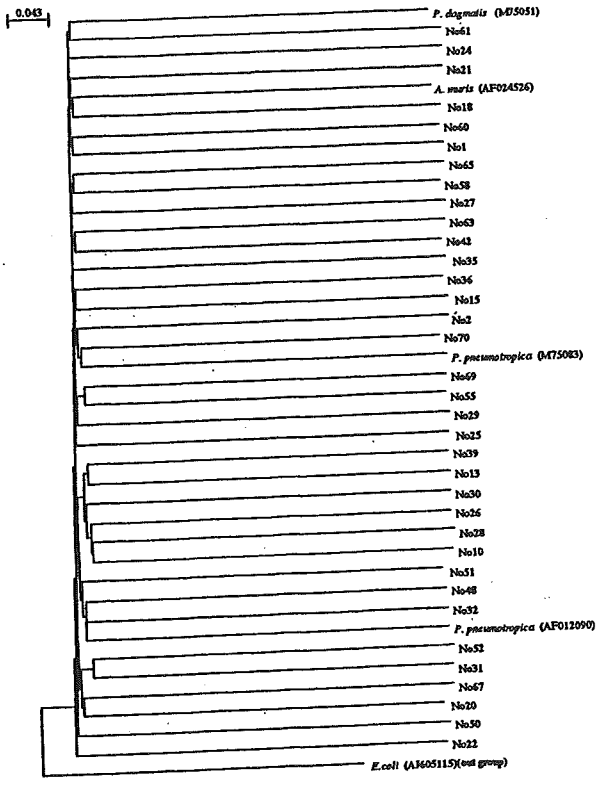

Fig. 1. Phylogenetic tree of tested isolates, *Pasteurella pneumotropica*, *Pasteurella dagmatis* and *Actinobacillus muris*. The scale bar represents a 0.043 difference in nucleotide sequence, as determined by measuring the lengths of horizontal lines connecting any two species. *P. pneumotropica* (M75083) is biotype Jawetz, *P. pneumotropica* (AF012090) is biotype Heyl.

separated into several clusters (Fig. 1). The taxonomical position of this organism is obscure. Genus Pasteurella is closely related to genus Actinobacillus as shown in Bergey's Manual 9th edition [9]. It may be very difficult to propose a simple identification procedure using a biochemical test or PCR test for this organism.

Second, from the standpoint of practical clinical microbiologists of laboratory animals, we would like to propose an identification procedure by which characters of the two biotypes are met and discrepancies with past identification results are minimized. Positive results for the two biochemical test kits were 61/69 (88%) and 39/69 (57%) in the ID and API 20 NE tests, respectively. The former is a kit for bacteria such as genus Haemophilus and Neisseria, and the latter is a kit for Gram-negative bacteria except enterobacteriaceae. The differences in target bacteria and the nutritional conditions in the two kits may cause the different results. The CIEA PCR test showed a wider detection range than the Wang PCR test, and the results for *P. pneumotropica* identification were basically similar to those of the ID test.

From these results, we recommend the following identification procedure for *P. pneumotropica*: colony observation on 5% horse blood agar plates, Gram-staining, the slide agglutination test with ATCC35149 antiserum for mouse, guinea pig, rabbit, and hamster isolates, and with MaR antiserum for rat isolates and a biochemical test using the ID test (Table 4). With this protocol 60 out of 69 isolates were identified as *P. pneumotropica*. However, one strain in 9 isolates not identified as *P. pneumotropica* showed 99% homology with the 16S rDNA sequence of biotype Jawetz.

There is no standardized identification procedure of *P. pneumotropica* in microbiological monitoring of laboratory animals in the U.S and Europe. Furthermore, the Federation of European Laboratory Animal Science Associations (FELASA) working group described that commercial identification kits do not identify *P. pneumotropica* properly [16]. Therefore,

Table 4. Proposed identification procedure of *P. pneumotropica*

Items	Results
Colony observation on 5% horse blood agar culuture for 48hr at 37°C	3–5 mm in diameter, smooth, low convex, non-hemolytic Jawetz: grayish-white, Heyl: yellow
Gram-stain	Gram-negative short rods or coccobacilli
Slide agglutination test with ATCC35149 anitisera for mouse, guinea pig and, rabbit and hamster isolates, MaR antisera for rat isolates	Agglutinated
Biochemical test with ID test HN 20 rapid	Identification as *P. pneumotropica* with % id 100

we think that our identification procedure of *P. pneumotropica* is useful for standardization between testing laboratories.

P. pneumotropica is considered an opportunistic pathogen in immunocompetent mice and it is recognized only as a factor worsening symptoms by co-infection with other murine pathogens, such as Sendai virus and *Mycoplasma pulmonis* [10]. The ICLAS Monitoring Center checked a total of 17,201 laboratory mice and rats in 2002, and found 508 mice contaminated with *P. pneumotropica*. A gross abnormality, a lung abscess, was found in only one of these contaminated animals and *P. pneumotropica* was isolated from the lung lesion. These results support the above discussion concerning the pathogenicity of *P. pneumotropica*. However, it was reported that *P. pneumotropica* caused orbital abscesses in immunodeficient mice [1,13], dual infection with the organism and *Pneumocystis carinii* caused severe symptoms in B cell deficient mice [11], and that mice with non-functional Toll-like receptor 4, such as C3H/HeJ and C57BL10/ScN, are susceptible to *P. pneumotropica* [2, 3, 8]. These results suggest that *P. pneumotropica* should be eliminated from immunodeficient mice and rats as well as mutant mice with non-functional Toll-like receptor 4.

The ICLAS Monitoring Center has proposed the following categorization of murine pathogens. Category A: Zoonotic and human pathogens carried by mice and/or rats. Category B: Fatal pathogens of mice and/or rats which can cause symptomatic disease and occasional deaths. Category C: Potential pathogens of mice and/or rats which usually cause asymptomatic infections accompanied by alterations of physiological functions. Category D: Opportunistic pathogens. Category E: Microbes as indicators of hygienic status of rearing environments. The ICLAS Monitoring Center has classified *P. pneumotropica* as category C in the categorization of murine pathogens. Based on its pathogenicity and the nature of the species with a wide diversity of characteristics, we propose changing the category of *P. pneumotropica* from C to D, so that the organism is eliminated from routine test items for microbiologic monitoring of immunocompetent mice and rats.

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