の感染はなかった.

ヒトが感染した場合，潜伏期は1～3週間程度で，ブルセラ症に特有の症状はない．軽症例では，風邪に似た症状を示す．発熱は，一定の発熱期と微熱あるいは平熱期を交互に繰り返す，いわゆる波状熱を示す．便秘，頭痛，悪寒，関節痛，筋肉痛，心内膜炎，肺炎，髄膜炎，リンパ節，脾臓，肝臓の腫脹，男性では精巣炎などもみられる．

*B. canis* に感染したイヌの多くは無症状であるが，長期間にわたり菌血症が続き，尿中に菌を排泄する．発症したイヌでは，発熱，削痩，関節炎のほか，妊娠末期に死流産を起こしたり，雄イヌでは精巣上体炎，精巣の萎縮がみられる．

ヒトは，病獣やその死流産胎児，分泌物，排泄物あるいはそれらに汚染された環境などからの感染に注意する必要がある．繁殖用のイヌで何度も死流産を起こしたり，なかなか受胎しない個体ではブルセラ症の検査を実施する．*B. canis* に感染したイヌでは尿中に排菌されるので，病獣の尿で汚染された環境は次亜塩素酸などによる消毒を励行する．

治療にはストレプトマイシン，テトラサイクリンが有効であるが，本菌は細胞内寄生性なので，宿主体内の菌を完全に殺滅するのは困難である．

**d. レプトスピラ症（四類感染症）**

ヒトではレプトスピラ症，動物ではレプトスピラ病と呼ぶ．病原体はらせん状のグラム陰性細菌 *Leptospira interrogans* で，両端あるいはその一端が鉤状に曲がっている（図 VIII.2.5）．本菌は多数の血清型（29群，250型以上）に分類されているが，わが国に分布するヒトに病原性のある主な血清型は，icterohaemorrhagiae（ワイル病），hebdomadis（秋疫B，七日熱），autumnalis（秋疫A），canicola（犬疫），australis（秋疫C），pyrogenes（東ワイル病）などである．

不顕性感染しているネズミ，イヌ，ウシ，ブタが多くみられる．わが国で分離される血清型と宿主となる動物の間には，イエネズミと血清型 icterohaemorrhagiae，野ネズミと grippotyphosa，イヌと canicola，ブタと pomona というような関係が認められている[4]．

ヒトや動物は不顕性感染したネズミ，イヌ，ウシ，ブタなどの尿で汚染した水，土などの自然環境から経皮感染する．感染した動物との接触によっても感染する．

わが国では，1999～2004年の間，毎年52～165頭のイヌのレプトスピラ病が報告されている．ヒトの症例は，2004年に18名，2005年に17名が報告されている．2005年には，静岡市のペット輸入販売業者の男性従業員2人が，輸入されたアメリカモモンガからレプトスピラ症に感染した事例があった[3]（表 VIII.2.8）．

ヒトの症状は，ワイル病のように黄疸，腎不全，内臓や皮下出血などの重篤な症状を呈するものから，ほとんど自覚症状のないものまで多様である．潜伏期は4日～2週間で，発熱，頭痛，眼結膜の充血，腎不全，髄膜炎，黄疸，出血などの症状がみられる．発熱に続き虹彩毛様体炎，硝子体混濁を起こすことがある．重症例では死亡することがある．

動物の潜伏期もヒトと同様である．イヌは全身感染を起こし，発熱，筋肉痛，口腔粘膜の出血，血便，腎炎，タンパク尿，黄疸などを示す．感染したイヌでは，長期間にわたり尿中に排菌するため，環境の汚染源，ヒトや他の動物への感染源となる．

本症の予防には，主な感染源となるネズミの駆除を


図 VIII.2.5 レプトスピラ（*Leptospira interrogans*）の電子顕微鏡写真（愛知医科大学 角坂照貴氏提供）

表 VIII.2.8 わが国のヒトレプトスピラ症およびイヌレプトスピラ病の発生状況

| 年次 | 症例数 | |
|---|---|---|
| | ヒト | イヌ |
| 1999 | 0 | 52 |
| 2000 | 0 | 63 |
| 2001 | 0 | 86 |
| 2002 | 0 | 165 |
| 2003 | 1 | 144 |
| 2004 | 18 | 158 |
| 2005 | 17* | 71 |

*アメリカモモンガから感染した2名を含む．

定期的に行うとともに，動物飼育施設を洗浄する際には，ゴム長靴とゴム手袋を着用する．レプトスピラは湿潤で中性ないし弱アルカリ性の環境で長期間生残するので，湿った環境はよく乾燥させた後，消毒を励行する．

ヒト，イヌともに感染を受けるおそれのある場合は，ワクチン接種が予防に有効である．特に，水遊びを好むレトリーバー種などの犬種にはあらかじめワクチンを打っておくとよい．

治療にはストレプトマイシン，カナマイシン，ドキシサイクリンが有効である．

**e. サルモネラ症**

サルモネラは *Salmonella enterica* および *Salmonella bongori* の2菌種に分類される．ヒトや動物に病原性を示すのは *Salmonella enterica* で，*S. enterica* はさらに6亜種（*enterica*, *salmae*, *arizonae*, *diarizonae*, *houtenae*, *indica*）に分類される．ヒトや動物から分離されるサルモネラのほとんどが亜種 *enterica* である．さらにサルモネラは血清型により約2500種類に分類される．

ペット，鳥類，爬虫類，両生類など，さまざまな動物がサルモネラを保菌しており，これらの動物やヒトなどの保菌者を仲立ちにして，自然界で循環している．ヒトは動物などの保菌者あるいはそれらによって汚染された食品，特に，食肉，卵，環境などから直接あるいは間接的に感染する．

サルモネラ食中毒は，わが国で最も多発する細菌性食中毒の一つで，毎年数百件の事例が報告されている（1998年：757件～2006年：124件）．学校，福祉施設，病院などで発生する大型の事例が多い．1980年後半から鶏卵関連食品を原因とする血清型 *S.* Enteritidis による感染事例が急増している[4]．海外では爬虫類などのペットを介する事例も増えている．わが国でも2004年にペットのイグアナを，2005年にはミドリガメ（ミシシッピアカミミガメ）を感染源とする幼児のサルモネラ症が発生している．

ヒトでは，感染12～24時間後に，吐き気，嘔吐，下痢，発熱（38℃前後）などの急性胃腸炎症状を起こす．サルモネラに感染した場合，健康な成人では胃腸炎程度の症状であるが，小児や高齢者では重症化し，死亡することもある．

動物の場合も幼獣では発熱，下痢を伴う全身症状を呈し，死亡する場合がある．成獣では発症することは少なく，多くは不顕性感染の型をとるが，まれに発熱，下痢を起こしたり，妊娠動物では流産することもある．

サルモネラ症は経口感染症なので，ペット動物に触れた後は手指をよく洗浄し，二次汚染を防ぐ．動物の糞便は適切に処理し，環境汚染を防止する．サルモネラを保菌した家畜由来の食肉，卵はサルモネラに時に汚染されていることがあるので十分な加熱・調理を行うことが予防上重要である．また，生肉をペットに餌として与えることは避けるべきである．

サルモネラ症の治療にはクロラムフェニコール，アンピシリン，ニューキノロン系の抗生物質が有効である．

**f. Q熱（四類感染症）**

偏性細胞内寄生性のグラム陰性細菌 *Coxiella burnetii* が原因である．本症が初めて報告された1935年当時，原因がわからなかったため，Query fever（不明熱）と命名されたのが，この病名の由来である．

ヒトは主に本菌を保菌している家畜の殺菌不十分な乳を飲んだり，排泄物によって汚染された粉塵やエアロゾルを吸入して感染する．ネコの流産胎児や胎盤が感染源になることがあるので，特にその取扱いには注意が必要である．

本症は世界各地で発生をみる．わが国では，1999～2005年の間，毎年7名（2004年）～47名（2002年）のQ熱患者が発生している．抗体陽性率は，わが国の乳牛では約30～40%，イヌで9.9～15%，ネコで14.2～41.7%，一般健康人で0.8～3.3%，野生鼠で約20%と報告されている．

ヒトが感染した場合，潜伏期は急性例で14～26日である．インフルエンザに類似した症状，すなわち突然発熱，頭痛，胸痛，筋肉痛，関節痛，悪寒，発汗などから気管支炎，肺炎，肝炎，髄膜炎，腎臓障害などをおこす．一般に予後は良好で，多くは2週間以内に回復する．

慢性例は急性例から移行することが多く，肝炎，心内膜炎，心筋炎，心外膜炎，壊死性気管支炎，骨髄炎を呈し，治療が遅れると死亡する場合がある．

イヌが *C. burnetii* に感染しても無症状から微熱を出す程度である．乳汁中に30日間，尿中に70日間も本菌を排出するといわれている．ネコの場合は，2～3日微熱が続いたり，菌血症が長期間続いたり，死産を起こすことがある[4]．

ヒトの慢性例では治療が遅れると死の転帰をとるので，Q熱を疑う症状の患者は，早期治療が重要である．ヒト，動物ともに治療にはテトラサイクリン系，ニューキノロン系の抗生物質が有効である．

動物は無症状のことが多く，また，糞便，尿中に本菌を長期間排泄するので，飼育環境を清潔に保つことが予防上重要である．特に，ネコが死流産を起こしたら，胎児や胎盤は直接手をふれずに廃棄するとともに，動物に対しては獣医師による適切な抗生物質治療を行う必要がある．海外では予防にワクチンが利用されているが，わが国ではない．

**g. オウム病（四類感染症）**

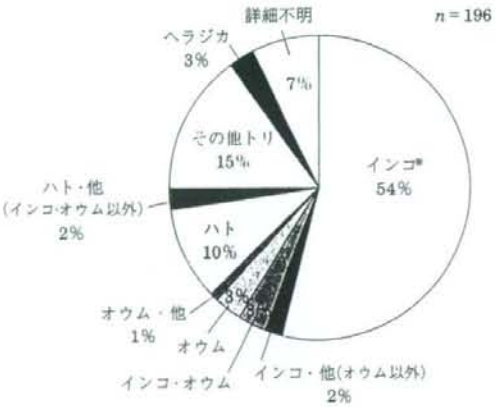
*Chlamydophila psittaci* が原因である．本菌は偏性細胞内寄生性のため，通常の細菌用培地では増殖しない．

オウム病は本来鳥類の疾病で，鳥類間では不顕性感染や持続感染の状態で病原体が維持されており，接触，吸入，経口感染する．発病期のオウム・インコ類は糞便，目やに，鼻水などの中に多量のオウム病クラミジアを排泄する．また，オウム病クラミジアは多数の哺乳類にも分布している．本菌に感染した哺乳類では，流産胎児や胎盤などに排菌される．また，回復した鳥や不顕性感染している鳥も長期間にわたり間欠的に糞便や鼻の分泌液中に菌を排泄し，感染源になる．ヒトは乾燥したオウム病クラミジアを含む塵埃の吸入により感染する．

わが国では，1999～2005年の間，毎年34名（2005年）～54名（2002年）のオウム病患者が報告されている．2001（平成13）年に川崎市営の動物公園で，シベリアヘラジカの出産に関わった職員5名に発熱を主な症状とした本症の発生があった．また，2001～02年にかけて，島根県松江市内の鳥の展示施設において職員4名，来園者12名にオウム病が集団発生した．

ヒトの潜伏期は1～2週間で，30～60歳の成人に多発する．小児では軽症の場合が多い．発熱，悪寒，頭痛，食欲不振，筋肉痛，関節痛などインフルエンザ様の症状が主で，下痢，便秘などもみられる．軽症例では無症状あるいは軽度のインフルエンザ様症状のみで経過するが，重症例では急性全身症状，異型性肺炎，心外膜炎，心筋炎，心内膜炎などを起こす[4]．

鳥類のオウム病クラミジアの保有率は，オウム目で最も高く，その他にスズメ目，ガンカモ目など多種の




図VIII.2.6 オウム病の推定感染源（1999年4月～2004年第53週）
※インコはセキセイインコ，オカメインコ，コザクラインコ，ルリコシボタンインコを含む．
（感染症情報センター感染症発生動向調査週報2005年第5号，速報より）

鳥類が本菌を保有している（図VIII.2.6）．鳥の多くは不顕性感染であるが，若齢鳥は特に感受性が高く，ストレスが加わると発症する．発病した鳥は元気消失，食欲不振，削痩，鼻腔からの漿液性・化膿性鼻漏などがみられる．緑色下痢便，結膜炎などを示すこともある．急性例では症状に気づかないまま死亡することもある．

多くのペット用鳥類がオウム病クラミジアを保菌していることを飼育者，輸入業者，繁殖業者に対し認識させるとともに，鳥を飼う際には十分な換気とこまめな排泄物の処理を心がける必要がある．また，鳥を新たに導入する際の検疫，死亡鳥の取扱い，健康鳥の定期的な検査と健康管理などが本症を予防するうえで重要である．

ヒト，動物ともにテトラサイクリン系，マクロライド系の抗生物質が有効である．

**h. エーリキア症**

*Ehrlichia* 属，*Anaplasma* 属のリケッチアが原因である．ヒトに病原性を示すエーリキアは *Ehrlichia chaffeensis*（ヒト単球エーリキア，主にアメリカに分布）および *Anaplasma phagocytophilum*（ヒト顆粒球エーリキア，主にアメリカとヨーロッパに分布）が知られている．イヌに感染するエーリキアは *Ehrlichia canis* および *Anaplasma platys* が世界的に分布しているが，アメリカでは *E. ewingii* および *E. chaffeensis* のイヌへの感染も報告されている．

いずれのエーリキアも，マダニの刺咬によりヒトやイヌに感染する．潜伏期は5～10日程度である．エーリキアに感染したヒトでは，頭痛，発熱，筋肉痛などのインフルエンザ様症状，悪心，嘔吐，下痢などの消化器症状，発咳などの呼吸器症状がみられる．

*E. canis* に感染したイヌでは，発熱，食欲不振，体重減少，リンパ節腫脹，脾腫，肝臓腫大，鼻汁漏出，呼吸困難，肺炎，髄膜脳炎，出血傾向など多様な症状を示す．急性期を過ぎると慢性化する．またイヌの *A. platys* 感染では周期性血小板減少症を示し，出血傾向がみられることがある．

本症はダニにより媒介されるので，流行地では忌避剤などを用いてダニの刺咬を避けることで予防する．

ヒト，動物ともにテトラサイクリン系，マクロライド系の抗生物質が有効である．〔丸山総一〕

## 文　献


1) Maruyama, S., Kabeya, H., Nakao, R., Tanaka, S., Sakai, T., Katsube, Y., Mikami, T. (2003): Seroprevalence of *Bartonella henselae*, *Toxoplasma gondii*, FIV and FeLV infections in domestic cats in Japan. *Microbiol. Immunol.* **47**, 147–153.
2) Maruyama, S., Izumikawa, K., Miyashita, M., Kabeya, H., Mikami, T., Yamanouchi, H., Sasaki, E., Yoshida, H., Izumikawa, K. (2004): First isolation of *Bartonella henselae* type I from a cat-scratch disease patient in Japan and its molecular analysis. *Microbiol. Immunol.* **48**(2), 103–109.
3) Masuzawa, T., Okamoto, Y., Une, Y., Takeuchi, T., Tsukagoshi, K., Koizumi, N., Kawabata, H., Ohta, S., Yoshikawa, Y. (2006): Leptospirosis in squirrels imported from United States to Japan. *Em. Infect. Dis.* **12**, 1153–1155.
4) 清水実嗣監修（2007）：人獣共通感染症，養賢堂．


## 2.3 ペットの原虫病と予防

ヒトや動物に寄生する原生生物を一般に原虫という．原虫は原則的に単細胞生物であるが，細菌と異なり真核生物で，複雑な寄生生活を営むものが多い．原虫を含む原生動物全体の分類は現在も目まぐるしく変化しているが，大まかに分けて，鞭毛虫類，肉質虫類，アピコンプレックス類，繊毛虫類に属するものがイヌやネコに寄生する．それぞれ多くの種の寄生が知られているが，ペットの主要な原虫病には以下のようなものがある．

### a. 鞭毛虫類

**（i） ジアルジア症**　*Giardia intestinalis* が病原体で，消化管に寄生する．体は扁平で，2個の核と4対の鞭毛をもつ．イヌ，ネコに対しては通常，強い病原性を示さないが，イヌでは，多数の扁平な虫体が腸管粘膜を覆ってしまうと，下痢や便秘を起こすことがある．ヒトも感染する（人獣共通原虫病）．感染は糞便中に排泄される耐久性をもつ嚢子（シスト）を経口的に摂取することによる．

**（ii） トリコモナス症**　*Pentatrichomonas hominis* が病原体で，ジアルジアと同様，消化管に寄生する．1個の核と波動膜をもつ1本の後鞭毛，5本の前鞭毛をもつ．下痢を起こしているイヌ，ネコの下痢便中にしばしば多数認められるが，本種は腸管内浮游生

図 VIII.2.7　*Giardia intestinalis* の電子顕微鏡写真
体は扁平で，腹面に吸盤と鞭毛をもつ．

図 VIII.2.8　*Pentatrichomonas hominis* の電子顕微鏡写真
体側に沿って走る鞭毛には波動膜がある．

特集

# 輸入齧歯類におけるバルトネラ属菌の保有状況ならびに分離株 *gltA* 遺伝子の比較系統解析

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泉　泰仁[☆1]　萩谷佳子[*1]　宇根有美[☆2]　吉川泰弘[*3]

## 要　約

近年，海外から輸入された多くの齧歯類が愛玩用として販売，飼育されている。本研究では，平成16年から18年に輸入された愛玩用齧歯類27種516頭について，バルトネラ属菌の保有状況の検討，ならびに分離株の *gltA* 領域における系統解析を行った。検討した齧歯類のうち17種142頭（27.5%）から本属菌が分離された。特に野外捕獲されたリス科の43.9%，ネズミ科の38.6%，トビネズミ科の43.2%，施設繁殖されたシマリスの16.7%が本属菌を保菌していることが判明した。さらに，輸入齧歯類には人に対し心内膜炎や視神経網膜炎の起因菌として考えられている菌種や，病原性が不明な新種と思われるバルトネラ属菌が広く分布していることが明らかとなった。

## はじめに

近年，住宅事情の変化あるいは愛玩動物に対する嗜好の多様化から，犬・猫のみならず様々な動物が家庭で飼育されるようになった。このような背景のもと，平成18年には，456,139頭の齧歯類が輸入，販売されており，その割合は哺乳類全体（475,224頭）の96.0%を占めている。その一方で，齧歯類は種々の人獣共通感染症の病原巣あるいは感染源となる場合があり，実際に，2005年に輸入されたアメリカモモンガを感染源とするレプトスピラ症も発生している。

齧歯類を自然病原巣とするバルトネラ属菌は10種2亜種が知られているが，そのうち *Bartonella elizabethae*, *B. grahamii*, *B. vinsonii* subsp. *arupensis*, および *B. washoensis* は，人に対し心内膜炎や視神経網膜炎などを起こすことが報告されている[1]。しかしながら，これまで輸入齧歯類における本属菌の保有状況を調査した報告はない。そこで本研究では，平成16年から18年に輸入された愛玩用齧歯類におけるバルトネラ属菌の保有状況について細菌学的および分子生物学的に検討するとともに，分離株の系統解析を行い，その公衆衛生学的意義について検討した。

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写真：知床国立公園　羅臼ビジターセンター前にて
毎年，長期休暇を利用して研究用のサンプリング（野鼠の捕獲）を行っています。昨年は，琵琶湖と知床半島に行くことができました。どちらも素晴らしい自然に囲まれての作業になりました。その土地の歴史や文化を知ることができるのも，サンプリングに行く楽しみのひとつです。

表1 わが国に輸入された愛玩用齧歯類のバルトネラ属菌の保有状況

| | 輸入地域 | 動物種 | 科 | 検査頭数 | 陽性頭数 (%) | 小計 (%) |
|---|---|---|---|---|---|---|
| **野生捕獲** | | | | | | |
| アジア | 中国 | ダウリアハタリス | リス科 | 10 | 4 (40.0) | 42/89 (47.2) |
| | 中国 | エゾリス | リス科 | 10 | 2 (20.0) | |
| | 中国 | シマリス | リス科 | 29 | 12 (41.4) | |
| | 中国 | タイリクモモンガ | リス科 | 10 | 5 (50.0) | |
| | タイ | バナナリス | リス科 | 30 | 19 (63.3) | |
| 北米 | アメリカ | アメリカアカリス | リス科 | 18 | 3 (16.7) | 27/68 (39.7) |
| | アメリカ | アメリカモモンガ | リス科 | 10 | 6 (60.0) | |
| | 不明 | コロンビアジリス | リス科 | 20 | 6 (30.0) | |
| | 不明 | リチャードソンジリス | リス科 | 20 | 12 (60.0) | |
| ヨーロッパ | オランダ | フトオアレチネズミ | ネズミ科 | 18 | 13 (72.2) | 13/47 (27.7) |
| | オランダ，チェコ | デグー | デグー科 | 29 | 0 (0.0) | |
| 中近東 | エジプト | アフリカチビネズミ | ネズミ科 | 20 | 0 (0.0) | 55/163 (33.7) |
| | エジプト | カイロトゲマウス | ネズミ科 | 31 | 3 (9.7) | |
| | エジプト | キンイロスパイニーマウス | ネズミ科 | 13 | 8 (61.5) | |
| | エジプト | ゼブラマウス | ネズミ科 | 11 | 0 (0.0) | |
| | エジプト | デブスナネズミ | ネズミ科 | 10 | 6 (60.0) | |
| | エジプト | シナイスナネズミ | ネズミ科 | 4 | 0 (0.0) | |
| | エジプト | フサオジャービル | ネズミ科 | 10 | 10 (100) | |
| | エジプト | オオエジプトアレチネズミ | ネズミ科 | 10 | 9 (90.0) | |
| | エジプト | オオミユビトビネズミ | トビネズミ科 | 16 | 13 (81.3) | |
| | エジプト | ヒメミユビトビネズミ | トビネズミ科 | 8 | 6 (75.0) | |
| | エジプト | ミミナガハリネズミ† | ハリネズミ科 | 10 | 0 (0.0) | |
| | パキスタン | ヒグミージェルボア | トビネズミ科 | 20 | 0 (0.0) | |
| | | | | | 小計 | 137/367 (37.3) |
| **施設繁殖** | | | | | | |
| アジア | 中国 | シマリス | リス科 | 30 | 5 (16.7) | 5/30 (16.7) |
| ヨーロッパ | オランダ | ステップレミング | ネズミ科 | 9 | 0 (0.0) | 0/99 (0.0) |
| | オランダ | フトオアレチネズミ | ネズミ科 | 10 | 0 (0.0) | |
| | オランダ | ゴールデンハムスター | ネズミ科 | 20 | 0 (0.0) | |
| | オランダ | ロボロフスキーハムスター | ネズミ科 | 10 | 0 (0.0) | |
| | オランダ，チェコ | ジャンガリアンハムスター | ネズミ科 | 30 | 0 (0.0) | |
| | オランダ，チェコ | デグー | デグー科 | 20 | 0 (0.0) | |
| 中近東 | パキスタン | ヒグミージェルボア | トビネズミ科 | 20 | 0 (0.0) | 0/20 (0.0) |
| | | | | | 小計 | 5/149 (3.4) |
| | | | | 合計 | 516 142 (27.5) | |

† ミミナガハリネズミは食虫目に属する。その他の動物は，齧歯目に属する。

## 輸入齧歯類におけるバルトネラ属菌の保有状況

平成16年から18年に輸入された野生捕獲あるいは施設繁殖された愛玩用齧歯類27種516頭を材料とした。血液培養の結果，17種142頭（27.5%）からバルトネラ属菌が分離された。輸入動物の由来別にみた保菌率は，野生個体が37.3%（136/367），繁殖個体が3.4%（5/149）であった。野生個体の輸入地域別にみた保菌率は，アジ

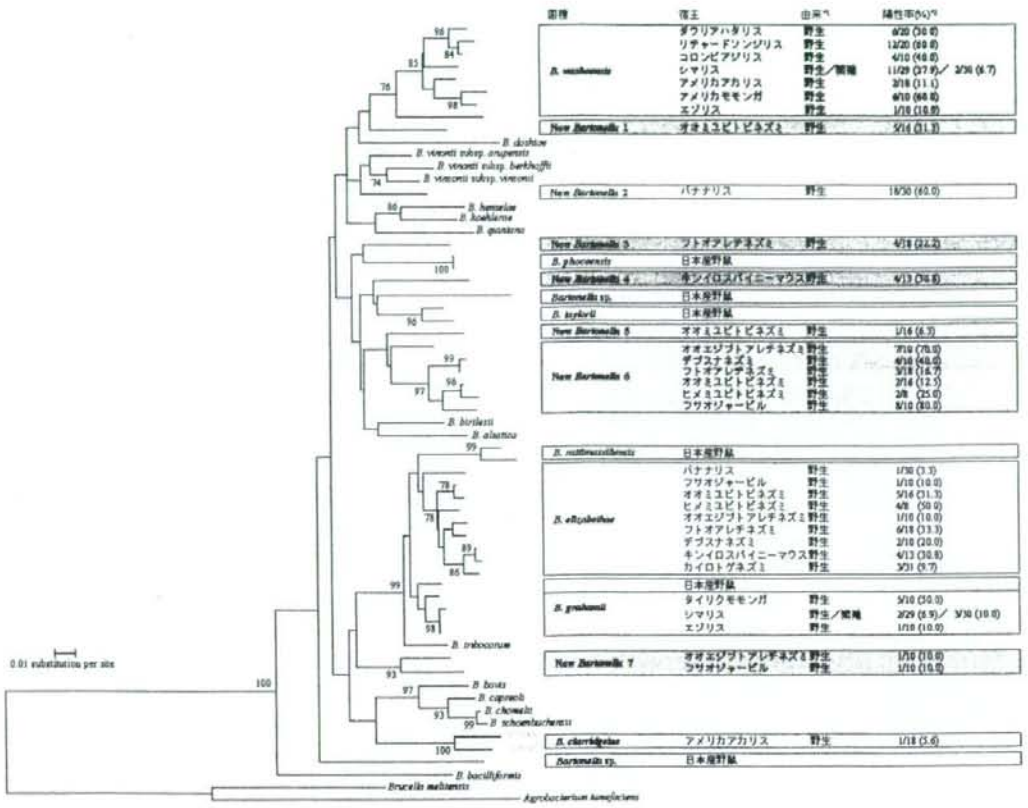


図 1　輸入齧歯類由来分離株，日本の野鼠由来分離株および既存のバルトネラ属菌の *gltA* 領域（312bp）に基づく系統樹
各分離株から得られた塩基配列は Kimura's 2-parameter distance 法により塩基置換の推定を行い，Neighbor-joining 法により系統樹を作成した。Bootstrap 解析は 1,000 回行い，bootstrap 値は，70% 以上を示す分岐にのみ記した。また，外群は *Brucella melitensis* 16M 株および *Agrobacterium tumefaciens* C58 株とした。
[1]：輸入動物の由来を示す（野生：野生捕獲個体，繁殖：施設繁殖個体）。
[2]：陽性頭数 / 検査頭数（%）を示す。

アの 47.2%（42/89），北米の 39.7%（27/68），中近東の 33.7%（55/163），ヨーロッパの 27.7%（13/47）であった。一方，繁殖個体では，中国のシマリス 16.7%（5/30）からのみ本属菌が分離された。動物種ごとでは，リス科動物の保菌率は，アメリカアカリスの 16.7%（3/18）～バナナリスの 63.3%（19/30）であった。ネズミ科動物の保菌率は，カイロトゲマウスの 9.7%（3/31）～フサオジャービルの 100%（10/10）であった。また，トビネズミ科では，オオミユビトビネズミの 81.3%（13/16）とヒメミユビトビネズミの 75.0%（6/8）から分離された。一方，デグー科およびハリネズミ科の動物からは分離されなかった（表 1）。

## 輸入齧歯類由来バルトネラ属菌のクエン酸合成酵素遺伝子に基づく系統解析

クエン酸合成酵素遺伝子（*gltA*）領域の塩基配列に基づく系統解析の結果，検討した分離株は，人の心筋炎，視神経網膜炎，心内膜炎，そして猫ひっかき病との関連が示唆されている *B. washoensis*，*B. grahamii*，*B. elizabethae*，*B. clarridgeiae* に近縁な 4 つのグループならびに，新種と思われる 7 つのグループに分類された。*B. washoensis* および *B. grahamii* 近縁株はいずれもリス科動物からのみ分

離されたのに対し，*B. elizabethae* 近縁株はリス科，ネズミ科，トビネズミ科の3科の動物から分離された。また，本来猫を自然宿主とする *B. clarridgeiae* に近縁な株がアメリカアカリスから初めて分離された。さらに，新種と思われる7グループに属する株のうち，New *Bartonella* 1～5はそれぞれ単一の動物種から分離されたのに対し，New *Bartonella* 6と7は，複数の動物種から分離された（図1）。

輸入齧歯類分離株とわが国の野鼠分離株を比較系統解析した結果，各genotypeはそれぞれ異なるグループに分類されたことから，輸入齧歯類に分布するバルトネラ属菌は，わが国の野鼠には未だ浸潤していないことが示唆された（図1）。

## おわりに

わが国に輸入された愛玩用齧歯類が高率（27.5%）にバルトネラ属菌を保菌していることが明らかとなった。また，分布している菌種は人に対して病原性のある4菌種と，病原性の不明な新種と思われる7グループのバルトネラ属菌であった。現在では，平成17年から動物の輸入届出制度が開始され，輸入動物の種類，数量だけでなく，動物毎に特定の感染症にかかっていない旨等を記載した証明書を届け出ることにより，輸入動物の現状が把握できるようになった。しかしながら，バルトネラ属菌を含む多くの病原菌の保菌状況を把握することは困難であるため，今後，輸入齧歯類の飼い主および動物取り扱い業等に対しては飼育や公衆衛生面での指導を行うとともに，動物の放逐により輸入齧歯類が保有するバルトネラ属菌がわが国の野鼠に浸潤することを未然に防止する啓発が重要であると思われる。


引用文献

1) Boulouis,H.J., Chang,C.C., Henn,J.B. et al. (2005) : *Vet. Res.* 36, 383-410.

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# Prolactin evokes lactational transmission of larvae in mice infected with *Toxocara canis*

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ABSTRACT

We investigated the trans-lactational maternal–neonatal transmission of *Toxocara canis* larvae in mice, with particular interest in the role of prolactin in their migration to the mammary gland. Two female mice were infected with 300 *T. canis* eggs soon after delivery of 27 offspring. After 1 week of breast-feeding, seven larvae were recovered from 4 of 13 offspring. After 2 weeks of lactation, 101 larvae were recovered from all the remaining offspring. Daily prolactin administration (5 μg) was performed 2 weeks before *T. canis* infection and continued until 2 weeks after infection in six non-pregnant female mice, which resulted in larval accumulation in the mammary gland. Furthermore, prolactin administration in female mice that had been infected with *T. canis* 4 weeks prior to prolactin treatment induced migration of larvae into the mammary gland. These findings suggest that prolactin is a promoting factor contributing to lactational transmission of *T. canis* larvae in mice.



## 1. Introduction

Human larval toxocariasis is a serious public health problem in many countries [1]. Adult worms of *Toxocara canis* parasitize the intestines of domestic dogs and wild carnivores, and the larval stage of the parasite opportunistically invades undefinitive hosts including humans, resulting in human larval toxocariasis [2]. The migration behavior of the larvae in undefinitive hosts has been well documented [3–5]. In mice, *T. canis* larvae begin to accumulate in the liver 2 days post-infection, and they continue to migrate via systemic circulation. Beyond the 10th day of infection, most have settled in the brain and muscle tissue [6–8]. The larvae found in skeletal muscle are encapsulated in granulomatous inflammatory tissue and can survive for a long period [4,8]; those in the brain tissue elicit minimal inflammatory response [4].

Furthermore, it has been established that trans-placental transmission is the major route for *T. canis* larvae migration from infected female dogs to puppies [9–13]. In mice, it has also been regarded that *T. canis* larvae are transmissible via placenta [14–16], although no previous studies demonstrated larvae from offspring. Recently, Reiterova et al. [17] observed that *T. canis* larvae in offspring from infected mother mice were recovered at the beginning of the 5th day post-delivery. Thus, lactational transmission rather than trans-placental migration was certainly a possible route of maternal–neonatal infection with *T. canis*. After infection, migrating larvae settle in skeletal muscle tissue, in which they are then arrested in granulomatous inflammatory tissue. A re-emergence mechanism for these arrested larvae during pregnancy, however, has yet to be identified. In the present study, we demonstrate that *T. canis* larvae are able to transmit from mother to neonate via the mammary gland, and that prolactin evokes lactational transmission of the arrested larvae.

## 2. Materials and methods

### 2.1. Animals

Conventional ICR mice and an inbred strain of BALB/c mice were purchased from CLEA Japan Inc., Tokyo. All experimental procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

### 2.2. Infections

*T. canis* eggs were obtained from the uteri of adult worms collected from naturally infected puppies after the administration of anthelmintics. Mature embryonated eggs were prepared following the method of Ohsima [5], and 300 eggs were inoculated into each mouse via a Teflon tube with a siliconized glass syringe [18].

### 2.3. Recovery of larvae

Each of the mammary glands and whole body of newborn mice were digested with artificial gastric juice (0.5% of 1:10,000 pepsin and 0.7% hydrochloric acid, pH 1.5) for 3 to 4 h with vigorous agitation. After centrifugation, the larvae in the sediment were counted using a stereoscopic microscopy on a microscope slide (7 × 14 cm). Examination

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**Table 1**
Numbers of larvae recovered from neonates

| Mother mouse | 7th day after birth | | | | 14th say after birth | | | |
|---|---|---|---|---|---|---|---|---|
| | Number of neonates examined | Number of neonates larvae recovered | Number of larvae/neonate | Total number of larvae recovered | Number of neonates examined | Number of neonates larvae recovered | Number of larvae/neonate[a] | Total number of larvae recovered |
| #1 | 5 | 4 | 1.4±0.5 (1–3)[a] | 7 | 6 | 6 | 9.5±0.8 (8–13)[a] | 57 |
| #2 | 8 | 0 | 0 | 0 | 8 | 8 | 5.5±0.9 (2–10)[a] | 44 |

Neonates were allowed to breast-feed from the mother mice, which were infected with 300 eggs of *T. canis* immediately after delivery.
[a] Mean ± SD (range).

of the brain was performed according to the method of Cho et al. [18]. In this experiment, we attempted to recover the larvae from skeletal muscle tissue by using the digestion method described above. However, the results were inconsistent in the number of larvae recovered from adult mice, because a large amount of sediments remained after digestion, making the counting of larvae using stereoscopic microscopy difficult. Therefore, we omitted the data on the muscle-stage larvae of the adult mice in this experiment.

### 2.4. Pathology of the mammary gland

Mammary glands of female mice were removed and fixed in 10% neutral formalin solution. Serial sections were then prepared and stained with haematoxylin and eosin. The degree of eosinophil infiltration around the mammary gland was estimated by the number of cells per square millimeter. We randomly selected 10 fields with a microscope of 100-fold magnification. To confirm cell identification, we observed at high magnification and counted the number of eosinophils. A careful attention was paid not to shift the original position.

### 2.5. Experimental design for trans-mammary transmission of larvae

Two pairs of 8-week-old ICR mice were mated in separate cages until the female mice became pregnant. Within 12 h after delivery, each of two female mice was infected with 300 eggs of *T. canis*, and then allowed to breast-feed their offspring for 2 weeks. The offspring were divided into two groups: one was killed on day 7 after delivery, the other was killed on day 14 after delivery. The number of larvae in the offspring was counted using the digestion method described above.

### 2.6. Effect of prolactin treatment in non-pregnant, infected mice

To investigate the effect of prolactin on the stimulation of larval migration from skeletal muscle or brain tissue, eight BALB/c female mice, at 8 weeks of age, were intraperitoneally injected with 5 μg of prolactin (100 mg/mL, Sigma, St. Louis, USA) in physiological saline everyday for 14 days, and were then infected with 300 *T. canis* eggs orally. Prolactin treatment was then continued for another 14 days. After treatment, the mammary glands were removed and the larvae were recovered. Two mice were used for histological purposes. As a control, seven additional mice were administered 0.5 mL of saline instead of prolactin.

**Table 2**
Effect of prolactin treatment in non-pregnant infected mice

| Treatment | Number of mice used | Number of mice larvae identified in mammary glands | Number of mice larvae identified in the brain | Number of larvae in mammary glands of identified mice | Number of larvae in the brain of identified mice |
|---|---|---|---|---|---|
| Prolactin | 6 | 6 | 6 | 9.8±3.5[a] | 36±16.3[a] |
| Saline | 5 | 0 | 5 | 0 | 34.4±24.2[a] |

[a] Mean±SD.

### 2.7. Effect of prolactin treatment in chronically infected mice

Six BALB/c female mice, at 4 weeks of age, were infected with 300 *T. canis* eggs. Four weeks later, 5 μg of prolactin was intraperitoneally administered everyday for 14 days. The mammary glands were then examined as described above. As a control, equal numbers of BALB/c mice were employed, and 0.5 mL of saline was injected into the peritoneal cavity everyday for 14 days.

### 2.8. Statistics

Statistical analysis was performed using Student's *t* test. *P* values of $<0.05$ were considered statistically significant.

## 3. Results

### 3.1. Larval transmission to neonates via mammary gland after birth

Two mother mice delivered 11 and 16 offspring, respectively. The offspring from each infected mother mouse, which were infected with *T. canis* within 12 h after delivery, were randomly selected and sacrificed on day 7 or day 14 after delivery. Table 1 presents the number of offspring infected and the number of larvae recovered on each of these days. The rate of infection in the offspring and the average number of larvae recovered were higher in the group sacrificed on day 14 compared with that sacrificed on day 7. Additionally, the total number of larvae recovered was significantly higher in the day-14 group ($P<0.05$).

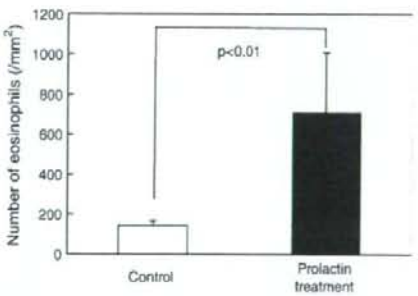


**Fig. 1.** Eosinophil counts around the capsules of mammary glands in mice. Solid bar, eosinophil count of prolactin-treated mice; open bar, that of untreated control mice. The mean number of eosinophils was 713.6±293.6 cells/mm² in the prolactin-treated group, and 144±21.3 cells/mm² in the saline-treated group. We randomly selected 10 fields with a microscope of 100-fold magnification. To confirm the cell identification, we observed at high magnification (×400) and counted the number of eosinophils. A careful attention was paid not to shift the original position.

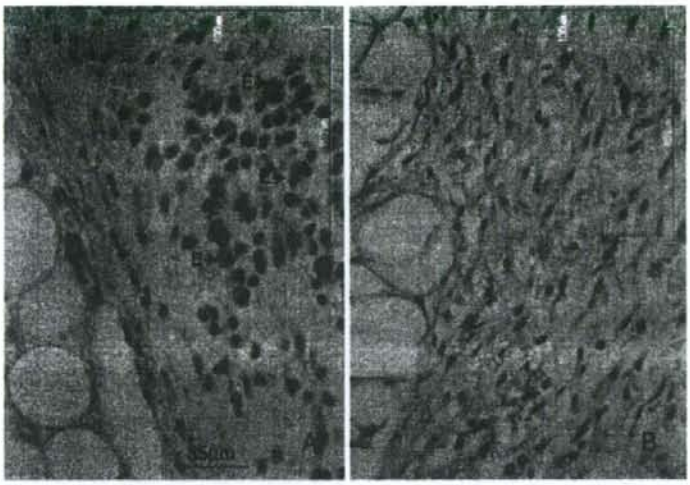

**Fig. 2.** Histopathological findings of mammary glands around the connective tissue in mice. Serial sections of mammary glands of female mice were stained with haematoxylin and eosin. Markedly higher eosinophilic (E) infiltrations around the connective tissue of the mammary gland were observed in the prolactin-treated mice (A) compared with the saline-treated mice (B).

### 3.2. Effect of prolactin on migration of larvae to the mammary gland

*T. canis* larvae were identified in the mammary glands of all infected mice, which were treated with 0.5 μg prolactin once a day intraperitoneally for 14 days before infection and 14 days after infection, although no larva was found in the control mice (Table 2). No significant difference in the number of larvae in the brain was observed between the prolactin-treated and saline-treated mice. These data suggest that prolactin might stimulate migration of larvae from skeletal muscle, the brain, or other organs to the mammary gland. Based on histological examination of 10 randomly selected fields, the eosinophil infiltrations around the capsule of the mammary gland were significantly increased in number in the prolactin-treated mice (713.6±293.6 6 cells/mm$^2$) compared with the saline-treated control mice (144±21.3 cells/mm$^2$, Figs. 1 and 2), suggesting that the inflammatory response against *T. canis* larvae was strong in the treated mice.

### 3.3. Effect of prolactin on chronically infected mice

Since administration of prolactin elicited a migration of larvae to the mammary gland, we next studied whether prolactin stimulates larval migration to the mammary glands from chronically infected mother mice in the absence of pregnancy. For this investigation, non-pregnant female mice, which had been infected with *T. canis* eggs 28 days previously, were administered prolactin for 14 days. Table 3 shows that larvae were recovered from the mammary glands in three of the four mice treated with prolactin, but no larva was found in the control mice. The number of eosinophils infiltrated in the mammary tissue was also significantly higher in the prolactin-treated group (Fig. 3).

In the prolactin-treated mice, glandular epithelial proliferation and dilatation of the ducts were observed, indicating a direct effect of prolactin against the mammary gland.

**Table 3**
Effect of prolactin treatment in chronically infected mice

| Treatment | Number of mice used | Number of mice larvae identified in mammary glands | Number of mice larvae identified in the brain | Number of larvae in mammary glands of identified mice | Number of larvae in the brain of identified mice |
|---|---|---|---|---|---|
| Prolactin | 4 | 3 | 4 | 3.8±1.9[a] | 51.3±15.1[a] |
| Saline | 4 | 0 | 4 | 0 | 49.8±5.7[a] |

[a] Mean±SD.

## 4. Discussion

In this study, we demonstrate that *T. canis* larvae are able to migrate from the mother to neonates through suckling behavior, and that this migration can be induced by the administration of prolactin. While trans-placental migration of the larvae from female dogs to puppies has been established [9–13], few studies have investigated maternal–fetal transmission of the larvae in mice. Lee et al. [16] found that the larvae migrated in the uterus and placenta from the 9th day of pregnancy, and in the fetus from the 11th day of pregnancy when mother mice were infected during pregnancy. In addition, they

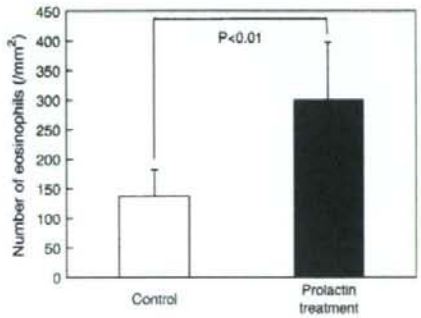


**Fig. 3.** Eosinophil counts around the capsules of mammary glands in chronically infected mice. Solid bar, eosinophil count of prolactin-treated mice; open bar, that of untreated control mice. The mean number of eosinophils was 300.8±95.6 cells/mm$^2$ in the prolactin-treated group, and 137.6±44.1 cells/mm$^2$ in the saline-treated group. Ten randomly selected fields at 100-fold magnification were observed via microscopy under a high magnification (400×).

identified larvae in the placenta and fetal blood vessels, histopathologically. They concluded that *T. canis* larvae were able to migrate through the placenta during pregnancy. However, because they did not examine the neonates after birth, they could not eliminate the possibility of trans-lactational transmission of the larvae from mother to neonates after delivery.

It is well documented that malaria infection induces placental injury, resulting in fetal loss in both humans and mice [19,20]. In murine toxocariasis, the litter sizes from infected mice are smaller than those from uninfected controls [21,22]. These data suggest that *T. canis* infection in mice can lead to mechanical injury of the placenta and a resultant decrease of litter size when the infection occurs during pregnancy.

Yet, in spite of these difficulties, newborns are still successfully delivered in most cases. In another previous study, larvae were found in offspring on day 5 after birth [15], suggesting suckling behavior might cause maternal–newborn transmission of *T. canis* larvae. In fact, our preliminary experiment revealed that larvae were first identified in offspring 11 days after birth (unpublished data). Thus, we hypothesized that larvae could migrate from mother to newborn mice through the mammary gland during suckling. The present findings support this hypothesis.

In general, *T. canis* larvae in mice settle in the brain and skeletal muscle after migration through the systemic circulation, and survive for a long period [4,8]. However, because we could not find any larvae in the mammary gland of non-pregnant infected mice, the larvae must be aroused by some sort of stimuli in order to migrate from those organs to the mammary gland. Prolactin, a lactogenic hormone, plays an essential role in the development of breast tissue. None of the non-pregnant mice not treated with prolactin showed the presence of larvae, in either the acute or chronic stage of infection, whereas prolactin-treated mice exhibited *T. canis* larvae infection in the mammary glands. One previous study discussed the relationship between *T. canis* infection and prolactin [23], reporting that the administration of prolactin led to a reduction in the number of larvae in infected mice. This may be related to the finding that prolactin acts as an immunomodulatory agent or proinflammatory cytokine in autoimmune diseases [24], and in several parasitic infections [25–28].

Eosinophil infiltration is a common feature in tissue-invading nematode infections, such as gnathostomiasis and trichinosis [29]. In toxocariasis, an eosinophilic granulomatous response is a typical pathological finding both in humans and in experimentally infected animals including mice [30,31]. Furthermore, eosinophil infiltration was demonstrated not only in the tissue adjacent to the larvae but also in that through which the larvae had passed [32]. These pathological changes are thought to be stimulated by the metabolic products from the larvae [29]. Therefore, we assumed that eosinophil infiltration around the capsule of the mammary gland in the prolactin-treated mice might be attributable to the migration of larvae into the mammary gland following stimulation of the tissue-arrested larvae.

The mechanism of this stimulation of tissue-arrested larvae during breast-feeding has yet to be elucidated. In hookworm infection, tissue-arrested larvae of *Ancylostoma caninum* were activated *in vitro* by TGF-β [33]. No such connection, however, has been demonstrated in *Toxocara* infection. The secretion of TGF-β is tightly regulated by the hormones estrogen and prolactin, and they are critical factors in the tissue-specific regulation of the local production of TGF-β in the mammary gland of the rat [34]. Therefore, we presumed that a similar cytokine reaction could be induced by prolactin, and may contribute to the reactivation of cryptic larvae in *Toxocara*-infected mice.

In the present study, we found clear evidence that prolactin is one of the factors in the lactational transmission of *T. canis* larvae from mother mice to offspring. Further investigation is needed to elucidate the precise mechanism of the stimulation of tissue-arrested larvae in mice.

## References


[1] Barriga OO. A critical look at the importance, prevalence and control of toxocariasis and the possibilities of immunological control. Vet Parasitol 1988;29:195–234.
[2] Gillespie SH. Human toxocariasis. J Appl Bacteriol 1987;63:473–9.
[3] Higashikawa H. Experimental studies on visceral larva migrans. Shikoku Acta Med 1961;17:1–20.
[4] Nichols RL. The etiology of visceral larva migrans. 1. Diagnostic morphology of infective second-stage *Toxocara* larvae. J Parasitol 1956;42:349–57.
[5] Oshima T. Standardization of techniques for infecting mice with *Toxocara canis* and observations on the normal migration routes of the larvae. J Parasitol 1961;47:652–6.
[6] Dunsmore JD, Thompson RC, Bates IA. The accumulation of *Toxocara canis* larvae in the brains of mice. Int J Parasitol 1983;13:517–21.
[7] Kondo K. Experimental studies on "larva migrans". J Kyoto Pref Univ Med 1970;79:32–56.
[8] Prokopic J, Figallova V. Migration of some roundworm species in experimentally infected white mice. Folia Parasitol (Praha) 1982;29:309–13.
[9] Augustine DL. Development in prenatal infestation of *Belascaris*. J Parasitol 1927;13:256–9.
[10] Douglas JR, Baker NF. Some host–parasite relationships of canine helminthes. In: McCauley JE, editor. Proc 26th Ann Biol Colloq; 1996. Corvallis: Oregon State University Press; 1996. p. 97–115.
[11] Fülleborn F. Askarisinfektion druck Verzehren eingekapselter Larven und uber gelungene intrauterine Askarisinfektion. Arch Schiffs-u Tropenhyg 1921;25:365–75.
[12] Scothorn MW, Koutz FR, Groves HF. Prenatal *Toxocara canis* infection in pups. J Am Vet Med Assoc 1965;146:45–8.
[13] Shillinger JE, Cram EB. Parasitic infestation of dog before birth. J Am Vet Med Assoc 1923;62:200–2003.
[14] Abo-Shehada MN, Herbert IV. The migration of larval *Toxocara canis* in mice. II. Post-intestinal migration in primary infections. Vet Parasitol 1984;17:75–83.
[15] Beaver PC, Jung RC, Cupp EW. Clinical Parasitology. 9th ed. Washington: Lee & Febiger; 1984.
[16] Lee KT, Min HK, Soh CT. Transplacental migration of *Toxocara canis* larvae in experimentally infected mice. J Parasitol 1976;62:460–5.
[17] Reiterova K, Tomasovicova O, Dubinsky P. Influence of maternal infection on offspring immune response in murine larval toxocariasis. Parasite Immunol 2003;25:361–8.
[18] Cho S, Egami M, Ohnuki H, Saito Y, Chinone S, Shichinohe K, Suganuma M, Akao N. Migration behaviour and pathogenesis of five ascarid parasites, *Toxocara canis*, *Bayfisascaris procyonis*, *B. transfuga*, *Ascaris suum*, and *A. lumbricoides* in the Mongolian gerbil, *Meriones unguiculatus*. J Helminthol 2007;81:43–7.
[19] Duffy PE, Fried M. Malaria in the pregnant woman. Curr Top Microbiol Immunol 2005;295:169–200.
[20] Poovassery J, Moore JM. Murine malaria infection induces fetal loss associated with accumulation of *Plasmodium chabaudi* AS-infected erythrocytes in the placenta. Infect Immun 2006;74:2839–48.
[21] Akao N, Desowitz RS, Kondo K. Decrease in litter size of female mice with *Toxocara canis*. Trans R Soc Trop Med Hyg 1990;84:724.
[22] Reiterova K, Tomasovicova O, Dubinsky P. Influence of *Toxocara canis* infection during pregnancy on offspring resistance towards re-infection. Parasitology 2006;132:625–33.
[23] Oshima T. Influence of pregnancy and lactation on migration of the larvae of *Toxocara canis* in mice. J Parasitol 1961;47:657–60.
[24] Matera L, Mori M, Geuna M, Buttiglieri S, Palestro G. Prolactin in autoimmunity and antitumor defence. J Neuroimmunol 2000;109:47–55.
[25] Benedetto N, Auriault C. Prolactin-cytokine network in the defence against *Acanthamoeba castellanii* in murine microglia [corrected]. Eur Cytokine Netw 2002;13:447–55.
[26] Di Carlo R, Meli R, Muccioli G. Effects of prolactin on rat paw oedema induced by different irritants. Agents Actions 1992;36:87–92.
[27] Mavoungou E. Interactions between natural killer cells, cortisol and prolactin in malaria during pregnancy. Clin Med Res 2006;4:33–41.
[28] Pearson RD. Prolactin and NK cells in maternal malaria. J Infect Dis 2001;184:662.
[29] Nawa Y, Abe T, Owhashi M. Host response to helminthes with emphasis on eosinophils and mast cells. In: Chowdhury N, Tada I, editors. Helminthology. New Delhi: Narosa Publishing House; 1994. p. 243–57.
[30] Kaplan KJ, Goodman ZD, Ishak KG. Eosinophilic granuloma of the liver: a characteristic lesion with relationship to visceral larva migrans. Am J Surg Pathol 2001;25:1316–21.
[31] Kayes SG, Oaks JA. Development of the granulomatous response in murine toxocariasis. Initial events. Am J Pathol 1978;93:277–94.
[32] Akao N, Kondo K, Sakai H, Yoshimura H. An immunopathological study of the liver of the mice infected with *Toxocara canis*. Jpn J Parasitol 1986;35:135–40.
[33] Arasu P. In vitro reactivation of *Ancylostoma caninum* tissue-arrested third-stage larvae by transforming growth factor-beta. J Parasitol 2001;87:733–8.
[34] Meli R, Gualillo O, Raso GM, Di Carlo R. Further evidence for the involvement of prolactin in the inflammatory response. Life Sci 1993;53:PL105–10.

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# An Improved Method for Recovery of Muscle-Stage Larvae From Mice Infected with *Toxocara canis*

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ABSTRACT: We report a modified digestion method that improves the recovery of *Toxocara canis* larvae from skeletal muscle. Minced muscle tissue from infected mice was incubated in artificial gastric juice for 48 hr at 37 C, and ethanol was added for the second 24 hr. This procedure allowed the larvae to be identified and counted more quickly than with the standard digestion method. This method allows measurement of the total number of larvae present in muscle tissue following oral intubation of embryonated eggs, although it does not permit counting of live larvae.

Following oral intubation of embryonated eggs, infectious-stage *Toxocara canis* larvae migrate into skeletal muscle tissue via systemic circulation. Muscle-stage larvae tend to increase in number after infection. Almost half of all recovered larvae enter skeletal muscles beyond the 10th day of infection (Oshima, 1961; Havasiova-Reiterova et al., 1995). These larvae are able to survive for long periods in muscle tissue. If an anthelmintic drug is effective against migrating larvae, the number of larvae appearing in skeletal muscle will be reduced. Therefore, for an anthelmintic trial, the number of muscle-stage larvae is a good indicator of efficacy (Fok and Kassai, 1998; Hrckova and Velebny, 2001; Horiuchi et al., 2005; Satou et al., 2005).

Both the Baermann technique and the digestion method using artificial gastric juice are used to detect larvae in skeletal muscle. The Baermann procedure, usually combined with a short-duration digestion method (less than 4 hr), permits the recovery of live larvae, but the extent of recovery is not satisfactory for estimating the total parasite burden. Additionally, since less than half of the skeletal muscle is usually employed for the digestion (Abdel-Hameed, 1984), the precise number of larvae recruited cannot be determined. In contrast, the digestion method alone permits a fairly good recovery, although a large amount of sediment remains after digestion, making the counting of larvae using stereoscopic microscopy quite time consuming. In the present report, we describe an improved method for recovering and counting larvae derived from skeletal muscle. The method is based on extended incubation in digestive fluid, followed by addition of alcohol.

Female BALB/c mice weighing 28–30 g were infected with 300 embryonated eggs of *T. canis* according to the method of Oshima (1961). Six mice were used for this experiment. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University. Three weeks later, skeletal muscle tissue from each mouse was minced with 150 ml of artificial gastric juice (0.5% of 1:10,000 pepsin and 0.7% hydrochloric acid, pH 1.5). After mixing well with a blender, the minced tissue was divided into 3 equal parts. The first portion was incubated in digestive fluid for 4 hr at 37 C with vigorous agitation. The mixture was then sieved with a wire mesh (mesh diameter: 1.0 mm), and the fluid was centrifuged at 320 *g* for 5 min. The total digestion time was 4 hr (method 1). Larval counting was performed on the resulting sediment using stereoscopic microscopy. Since undigested tissues remained on the mesh after sieving, these materials were re-incubated with digestive fluid for an additional 44 hr. They were vigorously agitated and prepared for counting in the same manner as before. The second portion of minced tissue was incubated in digestive fluid for 24 hr with vigorous agitation. The solution was centrifuged as before, and the sediment was re-incubated in 50 ml of fresh digestive fluid for an additional 24 hr. No filtration with wire mesh was performed. Thus, the total digestion time was 48 hr (method 2). Larval counts in the whole sediment were performed as before. The third portion was prepared in the same manner as the second portion, but 10 ml of 50% ethanol in distilled water was added to the sediment after the second 24 hr incubation step (method 3). The number of larvae in the sediment was then counted.

Table I shows the number of larvae recovered with each procedure. There was a significant difference in larval recovery between the 4-hr digestion group and the 48-hr digestion group ($p < 0.01$). Although ethanol treatment did not significantly affect recovery, we were able to find the larvae more easily in the ethanol-treated samples. The use of alcohol in the final step has the advantages that lipid droplets, which are insoluble in trypsin-based digestive fluid, are soluble in alcohol, and that alcohol acts as a surface-tension depressant that facilitates the identification of larvae. This is reflected in the time required to complete counting of a single sample: with ethanol treatment, counting took 16.7 ± 2.5 min (mean ± SD); without ethanol treatment, counting took 33.8 ± 7.5 min. For comparison, with the sample digested for 4 hr without ethanol, counting took 91.2 ± 14.1 min. From the undigested material, we were able to find larvae after additional incubation for 20 hr and 24 hr using freshly prepared digestive fluid, suggesting that a 4-hr incubation was insufficient for the digestion of skeletal muscle.

We further assessed whether this recovery technique can be carried out by an inexperienced person (T.N.). Six BALB/c female mice were orally administered albendazole (100 mg/kg/day) suspended in olive oil for 5 days, beginning 1 day before inoculation. Six control animals were given only olive oil. Three weeks after intubation, the mice were killed, and their skeletal muscle tissue was digested using method 3, under the guidance of an experienced researcher (Z.J.). Larvae migrating to the brain were counted by squash preparation (Abdel-Hameed, 1984). At the beginning of the experiment, it took almost 3 hr to complete the counting from just 1 skeletal muscle sample, but this soon fell to 30 min. The average recovery from skeletal muscle was 56.8 ± 4.8% in the control group versus 29.8 ± 9.8% in the albendazole group. In skeletal muscle, 104.5 ± 3.5 larvae were found in the control group versus 50.7 ± 22.3 in the albendazole group, indicating that prophylactic treatment can reduce the larvae in skeletal muscle (Table II).

The improved method described here requires substantially less operator time (since it is more than 5-fold faster) to count larvae, and the

TABLE I. Number of larvae recovered from skeletal muscle tissue of mice infected with 300 *T. canis* eggs.

| Digestion period (hr) | | | |
|---|---|---|---|
| 4 | | 48 | |
| | | Ethanol treatment | |
| Sediment | Undigested material | No | Yes |
| 9.5 ± 3.0 | 1 ± 0.9 | | |
| 10.5 ± 3.7 | | 23.2 ± 8.3* | 26.3 ± 8.5* |

Six mice were used for the experiment. Numbers are given as mean ± SD. Asterisk indicates a statistically significant increase in 48-hr incubation group versus 4-hr incubation group (Student's *t*-test, $P < 0.05$).

TABLE II. Number of larvae recovered from mice inoculated with 300 *T. canis* eggs.

| Albendazole* | | Control† | |
|---|---|---|---|
| Skeletal muscle | Brain | Skeletal muscle | Brain |
| 50.7 ± 22.3 | 38.8 ± 12.9 | 104.5 ± 3.5 | 66 ± 11.8 |

Larval recovery from skeletal muscle was performed using method 3.
* Six BALB/c mice were treated with 100 mg/kg/day of albendazole suspended in olive oil for 5 consecutive days beginning 1 day before inoculation.
† Six control mice were given only olive oil.

recovery is 3-fold higher than that of our previously reported methods (Horiuchi et al., 2005; Satou et al., 2005). However, the larvae recovered are no longer alive, which is likely due to the much longer incubation time required. Therefore, while this method would be suitable for measuring the efficacy of treatments that act before larval migration, it would not allow measurement of the active larval tissue burden.

## LITERATURE CITED


ABDEL-HAMEED, A. A. 1984. Effect of thiabendazole on the migration of *Toxocara canis* larvae in the mouse. Journal of Parasitology **70:** 226–231.

FOK, E., AND T. KASSAI. 1998. *Toxocara canis* infection in the paratenic host: A study on the chemosusceptibility of the somatic larvae in mice. Veterinary Parasitology **74:** 243–259.

HAVASIOVA-REITEROVA, K., O. TOMASOVICOVA, AND P. DUBINSKY. 1995. Effect of various doses of infective *Toxocara canis* and *Toxocara cati* eggs on the humoral response and distribution of larvae in mice. Parasitology Research **81:** 13–17.

HORIUCHI, A., T. SATOU, N. AKAO, K. KOIKE, K. FUJITA, AND T. NIKAIDO. 2005. The effect of free and polyethylene glycol-liposome-entrapped albendazole on larval mobility and number in *Toxocara canis* infected mice. Veterinary Parasitology **129:** 83–87.

HRCKOVA, G., AND S. VELEBNY. 2001. Treatment of *Toxocara canis* infections in mice with liposome-incorporated benzimidazole carbamates and immunomodulator glucan. Journal of Helminthology **75:** 141–146.

OSHIMA, T. 1961. Influence of pregnancy and lactation on migration of the larvae of *Toxocara canis* in mice. Journal of Parasitology **47:** 657–660.

SATOU, T., A. HORIUCHI, N. AKAO, K. KOIKE, K. FUJITA, AND T. NIKAIDO. 2005. *Toxocara canis*: Search for a potential drug amongst beta-carboline alkaloids—In vitro and mouse studies. Experimental Parasitology **110:** 134–139.

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ORIGINAL ARTICLE

# Evaluation of a rapid ELISA technique for detection of circulating antigens of *Toxoplasma gondii*

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**List of Abbreviations:** CAg, circulating antigen; CNS, central nervous system; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PcAb, polyclonal antibodies against soluble *Toxoplasma gondii* antigen.

**Key words**
circulating antigen, sandwich ELISA, *Toxoplasma gondii*, toxoplasmosis.

## ABSTRACT

**To evaluate a modified rapid ELISA method for detecting CAg during *Toxoplasma gondii* infection, we analyzed the specificity and sensitivity of the ELISA method by using experimental *Toxoplasma* infection in rabbits and also tested this method in human samples including 5428 serum, 548 cerebrospinal fluid and two breast milk samples. We prepared PcAb, and used it for rapid one-step sandwich ELISA testing in which an incubation time in the regular ELISA procedure was omitted. This method detected CAg at the concentration of 31.2 ng/mL, and no cross-reaction was found with antigens of protozoa (*Cryptosporidium parvum, Plasmodium falciparum*), trematode (*Schistosoma japonicum, Paragonimus* sp.) and nematode (*Brugia malayi, Ancylostoma duodenale, Ascaris lumbricoides* and *Trichinella spiralis*). CAg was detected in rabbit serum 3 days after infection, and optical density values reached a peak 9 ~ 13 days after infection, then declined gradually. Among human serum samples, the positive rate of CAg was 2.11% in cerebral paralysis patients, whereas it was 0.22% or 0.71% in patients without neurological symptoms or in uncomplicated pregnant women. The difference among these three groups was statistically significant ($P < 0.05$). The positive rate of cerebrospinal fluid samples from cerebral paralysis patients was 10.58%. There is a statistically significant difference between the positive rates of meat-packing workers and blood donors ($P < 0.01$). In the retrospective analysis, CAg was detected in accordance with the onset of clinical symptoms, suggesting that CAg could reflect the clinical course in humans. Together with these results, CAg detected in the modified rapid sandwich ELISA could be a sensitive marker for acute and active infection of *T. gondii*.**

Toxoplasmosis is caused by *Toxoplasma gondii*, an obligate intracellular protozoan parasite. It is one of the most epidemic parasitic diseases in human beings and animals, and infects as many as 35–40% of the adult population worldwide and demonstrates varying clinical manifestations. People are infected by inadvertent ingestion of oocysts or sporozoites in cat feces, or tissue cysts in undercooked infected meat or contaminated foods (1), and less frequently infected by direct recipience of tissue or blood from other contaminated humans, and vertical transmission from acutely infected mothers (2). Ingestion leads to the formation of tachyzoites acutely, which cause parasitemia and further dissemination and, along with the development of host immunity, parasites change to become the form of bradyzoites, which lead to latent infection with the formation of tissue cysts in skeletal muscle, heart muscle, and CNS tissue. Immunocompetent adults and adolescents with primary infection are generally asymptomatic,

but may include mild malaise, lethargy, and lymphadenopathy (3). Toxoplasmosis is an opportunistic disease, which is often lethal for immunocompromised patients such as those with AIDS, bone marrow or heart transplant recipients. According to surveillance, there are approximately 4000 persons with AIDS in the USA that become infected with toxoplasmic encephalitis every year, while up to 50% of all HIV-infected patients in Europe developed cerebral toxoplasmosis (4). Furthermore, toxoplasmosis may cause abortion or neonatal malformations such as birth defects, retinitis, and brain damage if acutely infected during pregnancy (5). To control toxoplasmosis effectively, efforts should be directed to develop a sensitive method for early diagnosis.

The most reliable diagnostic method for toxoplasmosis is detecting the pathogen, but this is costly, time-consuming and less sensitive. The ELISA detecting *Toxoplasma*-specific antibodies was found to be highly sensitive (92%) and specific (91%) when compared to the indirect immunofluorescent antibody test (IFAT), which was used as a reference test (6). Immunoglobulin (Ig) G antibody detection is widely used to diagnose *Toxoplasma* infection, but IgG antibody may be persistent lifelong among immunocompetent toxoplasma-infected individuals. The test lacks, therefore, reliability in the use for discrimination between recent and more distant infection. Moreover, it may be inconclusive in patients with underlying diseases causing suppressed antibody responses or in patients receiving immunosuppressive therapy (7). IgM class antibodies in patients with toxoplasmosis were shown to peak at 2 months, persisting for up to 6 months (8). Sometimes, the elevated levels of specific IgM can persist for at least several years. The presence of IgM alone is not always an indication of recent infection (9). Detection of IgM antibodies had a low predictive value for primary *T. gondii* infection (10). In recent years, a major effort has been made to diagnose recently acquired infection. Serum IgG avidity test and polymerase chain reaction (PCR) are two developed methods of this type. The IgG avidity test, for example, is useful as a confirmatory test along with other conventional serological tests. High avidity diagnosed past infection; however, persistence of low-avidity IgG reduced its value to differentiate acute and past infection (11) and, therefore, the IgG avidity test should not be used alone as a definitive test for decision-making (12).

*T. gondii* produces soluble antigens that are released into host tissues and the bloodstream (13). The detection of CAg might be useful for monitoring the disease. It may be more reliable than antibody detection because it directly reflects the viability and quantity of parasites in the host. Several laboratory procedures have been reported as a diagnostic means for detection of *Toxoplasma* antigen (i.e. countercurrent immunoelectrophoresis, agar gel immunodiffusion (14) and ELISA (15–17)). The detection of CAg can be helpful in the diagnosis of toxoplasmosis, especially in immunocompromised patients whose serological response can be impaired (18, 19). Various methods have been tested for the detection of CAg, such as an indirect ELISA (19), an ELISA using monoclonal antibodies (20), or avidin-biotin (AB)-ELISA (21). However, there is still a need to develop more easy, simple and accurate methods for detection of CAg. In the present research, we have developed a rapid and sensitive double-PcAb sandwich ELISA, which is capable of detecting *T. gondii* CAg in the early stage of infection.

## MATERIALS AND METHODS

### Preparation of soluble antigen of *T. gondii*

Soluble *Toxoplasma* antigens were prepared from the tachyzoites of the RH strain of *T. gondii* maintained in peritoneal cavities of mice that had been infected intraperitoneally (i.p.) with $3 \times 10^6$ tachyzoites. Briefly, the tachyzoites were purified by phytohemagglutinin (PHA) agglutinating filtration; then, the solution was microscopically detected for host cells to check the purity, the reduction rate of host cells should be above 98%. Then the tachyzoites were lysed by repeated freeze-thawing three times, followed by sonication. The suspension was centrifuged at 20 000 *g* for 30 min. The supernatant was collected and dialyzed overnight at 4 °C in PBS using a dialysis membrane with 6000 to 10 000 molecular weight apertures. Protein concentration was determined by the method of Coomassie brilliant blue G250 (22).

### Preparation of polyclonal antibodies

New Zealand white male rabbits, weighing approximately 2 kg, were used for immunization. Prior clearance from the local ethics committee was obtained. Soluble *T. gondii* antigens (0.1 mL) of *T. gondii* combined with 0.1 mL complete Freund's adjuvant were injected into each rabbit by intramuscular (i.m.) injection at multiple sites, followed by three subsequent boosts at 8-day intervals with antigens combined with incomplete Freund's adjuvant. The total dose of immunization for each rabbit was 4–5 mg/kg. Then, antiserum was collected from an ear vein and checked once a week after the final booster dose. When the detected level of the antibody became stable at 1:2048 in indirect hemagglutination assay (IHA; Institute of Biological Products, Lanzhou, China), sera were collected from the hearts of the rabbits. The collected serum was treated with 50% saturated ammonium sulfate. The

precipitate was dissolved in PBS (pH 7.2) and treated with 33% saturated ammonium sulfate three times. After centrifugation, the precipitate was dialyzed overnight against the same phosphate solution at 4 °C. The IgG fraction (PcAb) was prepared from this profractionate solution with DEAE iron-exchange column chromatography, avoiding major interference with host components. Part of the PcAb extracted from rabbit serum was conjugated with horseradish peroxidase (HRP) according to a standard $NaIO_4$ conjugating method (23).

### Double-PcAb sandwich ELISA

A double-PcAb sandwich ELISA was performed in microtitration trays. In brief, polystyrene microtitration plates (Costar ELISA 96-well plates [Corning Glass Works, Corning, CA, USA]) were coated were coated with 100 μL rabbit PcAb and then incubated overnight at 4 °C. The plates were drained and washed three times with 0.1 M PBS containing 0.05% Tween 20 (PBST) for 1 min each time. With highly activated HRP-PcAb and sufficient washing, the blocking step is not necessary in this double-sandwich ELISA. Soluble *T. gondii* antigens or test samples (100 μL) were added to the wells. At the same step, 50 μL HRP-conjugated PcAb as a detecting reagent was added and the plates were incubated at 37 °C for 1 hr. After washing five times as described above, 100 μL of substrate, tetramethylbenzidine solution, was added and incubated at room temperature for 20 min, and the reaction was stopped with 50 μL of 2 M $H_2SO_4$ per well. The optical density (OD) was read at 450 nm using a plate reader (Huadong Electronics, Hangzhou, China). All samples were tested in duplicate to give average absorbance values. The most appropriate experimental condition such as coating concentration and the dilution of samples and PcAb-HRP were determined previously by the chessboard titration method (24). S/N (OD value of samples/OD value of negative control) was calculated for each, and samples with S/N 2.1 were judged as positive. As a background reaction, 15 sera from healthy humans were mixed and used as a negative control for the clinical test.

### Cross-reaction and detectable limit of the method

Crude antigens of *Cryptosporidium parvum*, *Schistosoma japonicum*, *Paragonimus* sp., *Brugia malayi*, *Ancylostoma duodenale*, *Ascaris lumbricoides* and *Trichinella spiralis* were prepared for specificity analyses. The specificity of the ELISA for detection of *T. gondii* CAg was evaluated by analysis of cross-reaction to the unrelated parasite antigens shown above. Detectable limitation of the method was determined by the serial antigen dilution.

### CAg kinetics in rabbits infected with *T. gondii*

In total, 10 New Zealand white rabbits were peritoneally injected with $10^5$ tachyzoites of RH strain and were bled from an ear vein at different time points during 1–90 days after infection. Sera from five rabbits without *Toxoplasma* infection were also collected as negative controls. Circulating antigens present in rabbit sera were determined using the double-PcAb sandwich ELISA method. Kinetic patterns of CAg within 90 days after infection were analyzed.

### Detection of CAg in human samples

Clinical serum samples were obtained from 5147 donors: 3598 outpatients with suspected parasitic infections, 322 cerebral paralysis cases, and 1227 normal pregnant women. We also obtained 548 cerebrospinal fluid (CSF) samples from patients with CNS symptoms. The possibility of cerebrovascular diseases, cerebral hemorrhage or infarction was excluded by clinical diagnoses. Patients with clinical symptoms like fever, lymph node swelling or jerks with unknown origin, were regarded as parasitic infection-suspected cases. Two milk samples from breast-feeding women with serum CAg positive were obtained. Serum samples from 161 meat-packing workers in a slaughter-house and 120 blood donors without any clinical symptoms were collected for CAg detection. These samples were stored at −20 °C until they were tested for CAg by the ELISA method. All human samples were collected under the approval of the Regional Committee for Ethics and Research, Zejiang Province, China.

### Comparative study of double-PcAb sandwich ELISA with other detection methods

To compare the efficacy of double-PcAb sandwich ELISA with other detection methods, 36 CAg-positive serum samples were selected and tested for detecting *T. gondii* conducted by microscopic examination or through inoculation and serial passages in ICR mice. For microscopic examination, each sample was dropped and smeared on five slides and detected for tachyzoites and cysts by two trained laboratory technical staff. For inoculation examination, each sample was i.p inoculated into three ICR mice; after serial passages, the peritoneal lavage fluid of the mice was microscopically detected for tachyzoites and cysts. Five CAg-seronegative serum samples were also i.p. injected into mice for the control test. Two hundred and ninety-nine amniotic fluid samples from IgG-seropositive pregnant women were detected for *Toxoplasma* infection both by double-PcAb sandwich ELISA and PCR (PCR diagnostic kits were provided by The Second Military Medical

University, China). PCR was performed according to the protocol. Briefly, PCR was performed using an initial denaturation at 95 °C for 5 min, 35 cycles of 94 °C for 40 s, 55 °C for 30 s, and 72 °C for 60 s. This was followed by a final extension at 72 °C for 7 min. After amplification, 15 $\mu$L of the products was electrophoresed with 2% agarose gel, stained with ethidium bromide and visualized with a UV transilluminator.

We tested CSF samples from 29 clinical cases with CNS symptoms by IHA and ELISA to detect mean *Toxoplasma*-specific CAg, IgG or IgM antibodies during 3 to 21 days after the onset of the disease. For IHA, samples were diluted using two-fold serial dilutions from 1:16; each 50 $\mu$L diluted serum was mixed with 25 $\mu$L sensitized red cells in wells of a U-bottomed plate. Positive and negative controls were included on each plate. Plates were shaken mechanically for 15 to 30 s, and incubated at room temperature for 1 hr. Hemagglutination patterns were read independently by two trained laboratory technical staff; no agglutination was negative, > 2+ agglutination from 1:64 dilution was interpreted as positive. Capture ELISA was conducted to detect IgM antibody. Briefly, the wells of microtitration plates were coated overnight at 4 °C with 100 $\mu$L sheep anti-human IgM to *T. gondii*. After washing, 100 $\mu$L serum samples at 1:20 dilution were added to each well and incubated for 0.5 hr at 37 °C. After washing, 100 $\mu$L soluble antigen and PcAb-HRP were added to each well and incubated for 0.5 hr at 37 °C. After washing and addition of the substrate, the reaction was stopped and read at 450 nm. Indirect ELISA was applied to detect IgG antibody. The wells of microtitration plates were coated overnight at 4 °C with 100 $\mu$L of soluble antigen of *T. gondii*. After washing and blocking, 100 $\mu$L serum samples at 1:100 dilution were added to each well and incubated for 0.5 hr at 37 °C. After washing, 100 $\mu$L sheep anti-human IgG-HRP were added to each well and incubated for 0.5 hr at 37 °C. After washing and addition of the substrate, the reaction was stopped and read at 450 nm.

### Statistical analysis

Statistical analyses were done by Student's *t*-test. Statistical significance was determined when *P* values were less than 0.05.

## RESULTS

### Cross-reaction and detectable limitation of the method

As shown in Figure 1, the method could detect as low as 31.2 ng/mL with an OD value of 0.24, whereas the OD value of the negative control was 0.1. The S/N value in this case was > 2.1.

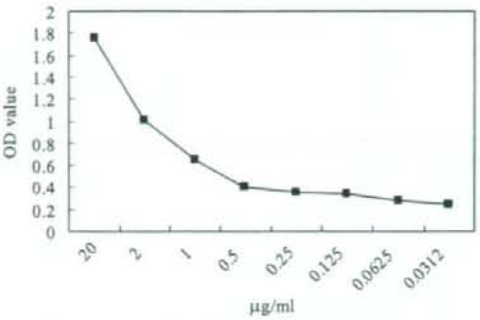


**Fig. 1.** Sensitivity of the ELISA to detect CAg of toxoplasma. The minimum antigen concentration in this method is 0.0312 $\mu$g/mL.

There was no cross-reactive parasite antigens tested: all the OD values for those crude antigens of *Cryptosporidium parvum*, *Schistosoma japonicum*, *Paragonimus* sp., *Brugia malayi*, *Ancylostoma duodenale*, *Ascaris lumbricoides* and *Trichinella spiralis* were less than 0.10. When we tested human serum from patients with *Falciparum* malaria, no positive response was observed, indicating no cross-reaction with antigens of *Plasmodium falciparum* (data not shown).

### Kinetics of CAg in infected rabbits

CAg was detected on day 3 after infection (S/N = 2.11), and then the OD value increased markedly from 5 days after infection. The highest OD value was observed on day 9 (S/N = 22.13), thereafter the values decreased gradually. On day 20 after infection, CAg was still detectable with comparatively low OD value, and positive results lasted until day 90 (Fig. 2). No positive result was found in any serum samples of the five rabbits without *T. gondii* infection.

### Detection of CAg in human clinical samples

To evaluate the double-PcAb sandwich ELISA for detecting CAg in clinical cases, we tested human serum and CSF samples. As shown in Table 1, the positive rate of CAg in sera from patients with cerebral paralysis was 2.11%, whereas in outpatients without neurological symptoms it was 0.22%, or 0.71% in sera from pregnant women without complications. There was significant difference among these three groups ($P < 0.05$). Of 548 CSF samples, 58 (10.6%) were positive. One of two milk samples, collected from CAg-positive women during serum testing, was CAg positive. Although vertical transmission from

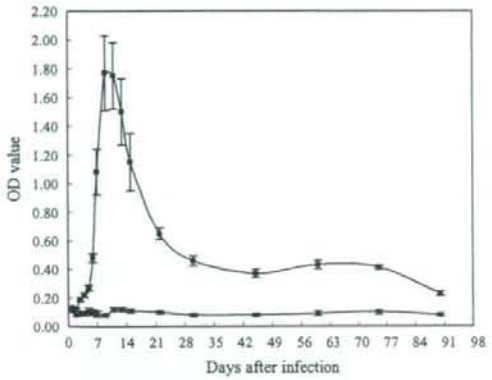


**Fig. 2.** Kinetics pattern of CAg in infected rabbits. Three days after infection, CAg could be detected. At 9 days after infection, the optical density (OD) value was highest and then decreased. In the later phase from 20 to 90 days after infection, CAg could still be detected at low but significant levels. Ten infected rabbits and five control rabbits were used for the lozf. ♦, Control group; ■, infected group.

breast-feeding has not been demonstrated in humans (5), CAg positivity found in breast-feeding milk in the present study suggest that populations of breast-feeding women should be notified to avoid vertical infection. Fifteen samples of meat-packing workers were CAg positive, the positive rate was 9.3%. One sample from a blood donor was CAg positive, the positive rate was 0.8%. There is a statistically significant difference between the positivity rates of meat-packing workers and blood donors ($P < 0.01$); the result indicated that the infection of *Toxoplasma* was highly related to frequently handling meat products. Also, one CAg-positive case in blood donors alerts us to the existing risk of *Toxoplasma* infection through blood transfusion. (Table 2).

## Comparison between the sandwich ELISA and other techniques for diagnosis of toxoplasmosis in human samples

Of CAg-positive samples, we randomly selected 36 samples for further examination to detect the pathogen by inoculating mice. All five CAg control samples were negative with inoculation examinations. Among the 36 samples tested, 21 were found to be *T. gondii* positive by inoculation examinations, and the coincidence rate between the two methods was 58.3%, although only three were positive by direct microscopic examination. The result indicated that the double-PcAb sandwich ELISA for detection of CAg was sensitive and of considerable accuracy (Table 2). In 12 CAg-positive samples, 11 samples were PCR positive, the accordance rate was 91.67%. All of these 12 cases showed abnormal childbirth by follow-up visit. There were 21 positive cases detected by PCR which resulted in negative results, whereas double-PcAb sandwich ELISA for CAg were used (Table 3). But the 5-year follow-up visit showed no abnormality in the offspring of the above 21 cases, indicating the possibility of false-positive PCR; double-PcAb sandwich ELISA showed more accuracy compared to PCR.

**Table 1** Detection for CAg from different human samples

| Sample sources | Origin | No. cases | No. positives | Positive rate (%) |
|---|---|---|---|---|
| Serum | Parasitic infection suspected | 3598 | 8 | 0.22 |
| Serum | Cerebral paralysis cases | 322 | 7 | 2.11 |
| Serum | Pregnant women | 1227 | 8 | 0.71 |
| Cerebrospinal fluid | Patients with CNS disease | 548 | 58 | 10.58 |
| Milk | CAg seropositive | 2 | 1 | 50 |
| Serum | Meat-packing workers | 162 | 15 | 9.3 |
| Serum | Blood donors | 120 | 1 | 0.8 |

Clinical samples and samples from meat-packing workers and blood donors were detected for *Toxoplasma* infection by double-PcAb sandwich ELISA.

**Table 2** Detection of CAg by sandwich ELISA and isolating pathogens from different clinical human samples

| Sample | Source of sample | No. cases CAg (+) | No. cases +ve for inoculating mice | No. cases +ve for microscopic examination |
|---|---|---|---|---|
| Blood | Outpatients | 4 | 2 | 0 |
| Blood | Cerebral paralysis cases | 1 | 0 | 0 |
| CSF | Patients with CNS disease | 30 | 18 | 3 |
| Milk | Breast-feeding women | 1 | 1 | 0 |

Clinical samples of CAg seropositivity were etiologically detected by microscopic and inoculating examinations.

**Table 3** Results of PCR and CAg ELISA in amniotic fluid samples

| | CAg(+) | CAg(−) |
|---|---|---|
| PCR(+) | 11 | 21 |
| PCR(−) | 1 | 266 |

Total of 299 amniotic fluid samples from IgG-seropositive pregnant women were detected both with PCR and double-PcAb sandwich ELISA. PCR, polymerase chain reaction.

In order to assess the CAg detection method with antibody detection methods, CSF samples from 29 clinical cases with CNS symptoms retrospectively suspected of having acute toxoplasmosis (whose samples in the time course were stocked) were selected for testing by IHA and by ELISA detecting CAg, IgG and IgM antibodies. These 29 patients showed symptoms such as seizure, vomiting and/or high fever, but routine examination of CSF ruled out the possibility of purulent or bacterial encephalitis. CAg could be detected on day 3, coinciding with the onset of clinical symptoms, whereas IgM and IgG were detected 8 and 21 days after the appearance of clinical symptoms, respectively. The whole positive rate of CAg detection by the sandwich ELISA was 89.7% (26/29), whereas the results of IHA was 13.8% (4/29). The difference in the positive rate between CAg-ELISA and IHA was of statistical significance ($P < 0.01$) (Table 4).

## DISCUSSION

Toxoplasmosis is widely distributed throughout the world. The positive rate in the Chinese population is approximately 4–9% in serological diagnosis detecting specific IgG antibodies; in abattoirs, the positive rate is up to 63.4%. In animals, the positive rate of pigs in an endemic zone reaches 52.8%. There was significant association between *Toxoplasma* infection and dystocia of live pigs (25). Considering the highly epidemic situation of toxoplasmosis in China and harm to health and the local economy, it is important to develop an accurate detection method for the parasite infection in the early phase.

As a method for early diagnosis, a PCR-based approach has been developed. Recent studies have shown that testing of PCR amniotic fluid is useful for identification or exclusion of fetal *T. gondii* infection (3). Lewis *et al.* reviewed the clinical, diagnostic and therapeutic aspects of toxoplasmosis in the setting of hematopoietic stem cell transplantation and the role of a PCR-based testing for early detection and diagnosis (26). However, a report from another laboratory showed that PCR does not have sufficient sensitivity for *Toxoplasma* infection (27). Furthermore, PCR detection is not suitable for monitoring and survey in mass groups.

The ELISA test for demonstrating antigenic fractions of the parasite in the host's circulation allows a parasitological diagnosis with higher sensitivity than the methods for detecting the whole parasite (28). We established a simple double-PcAb sandwich ELISA method to detect CAg in sera and body fluid samples, which permits a rapid detection throughout of specimens because samples for examination and peroxidase-conjugated antibodies were added to the plate in one step, removing an incubation stage. This method was sensitive, as it could detect CAg at a minimum concentration of 31.2 ng/mL. It is simple enough to operate in mass survey.

A notable point for the detection method is its applicability for early diagnosis, because the specific CAg in infected rabbits could be detected as early as 3 days after infection. The titer is highest at 9 days after infection, and the kinetic pattern of CAg coincided with the symptoms in rabbits. The infection experiment in rabbits revealed that there were no obvious symptoms during 3 days after

**Table 4** Results of immunological tests for toxoplasmosis on different days after onset of the disease

| Days after onset of disease | No. cases | IHA (+) (%) | IgG (+) (%) | IgM (+) (%) | CAg (+) (%) |
|---|---|---|---|---|---|
| 3 | 1 | 0 (0) | 0 (0) | 0 (0) | 1 (100) |
| 4 | 2 | 0 (0) | 0 (0) | 0 (0) | 2 (100) |
| 5 | 3 | 2 (67) | 0 (0) | 0 (0) | 2 (67) |
| 6 | 3 | 0 (0) | 0 (0) | 0 (0) | 3 (100) |
| 7 | 2 | 0 (0) | 0 (0) | 0 (0) | 2 (100) |
| 8 | 3 | 0 (0) | 0 (0) | 1 (33) | 3 (100) |
| 9 | 1 | 0 (0) | 0 (0) | 1 (100) | 1 (100) |
| 10 | 5 | 2 (40) | 0 (0) | 1 (20) | 5 (100) |
| 14 | 6 | 0 (0) | 0 (0) | 1 (17) | 5 (83.3) |
| 21 | 3 | 0 (0) | 1 (33) | 1 (33) | 2 (67) |
| Total | 29 | 4 (13.8) | 1 (3.4) | 5 (17) | 26 (90) |

Clinical patients were serologically detected with IHA, capture ELISA for IgM, indirect ELISA for IgG, and double-PcAb sandwich ELISA for CAg on different days after onset of disease. Ig, immunoglobulin; IHA, indirect hemagglutination assay.