Use of a real-time polymerase chain reaction-based fluorogenic 5' nuclease assay to evaluate insect vectors of *Corynebacterium pseudotuberculosis* infections in horses

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Objective—To develop and use a sensitive molecular assay for detecting the phospholipase D (PLD) exotoxin gene of *Corynebacterium pseudotuberculosis* in an attempt to identify insect vectors that may be important in transmission of clinical disease in horses.

Sample Population—2,621 flies of various species.

Procedure—A real-time polymerase chain reaction (PCR)-based fluorogenic 5′ nuclease (TaqMan) system (ie, TaqMan PCR assay) was developed for the detection of the PLD gene in insects. Flies were collected monthly (May to November 2002) from 5 farms in northern California where *C pseudotuberculosis* infection in horses is endemic. Three of the 5 farms (which housed a total of 358 horses) had diseased horses during the study period. A total of 2,621 flies of various species were tested for the PLD gene of *C pseudotuberculosis*.

Results—Evidence of bacterial DNA for the PLD gene was detected in skin biopsy specimens from clinically affected horses and from 3 fly species collected from farms where affected horses were housed. Farms with a high incidence of diseased horses had a high proportion of insects carrying the organism. High percentages of flies with positive results for the PLD gene were observed in October, when most clinically affected horses were observed.

Conclusions and Clinical Relevance—Our results are consistent with the hypothesis that *C pseudotuberculosis* may be vectored to horses by flies. Three potential vectors were identified, including *Haematobia irritans, Stomoxys calcitrans,* and *Musca domestica.* The organism can be identified in up to 20% of house flies (*Musca domestica)* in the vicinity of diseased horses. (*Am J Vet Res* 2004;65:829–834)

*C*orynebacterium pseudotuberculosis is a pleomorphic, facultative intracellular, gram-positive rod that causes 3 distinct disease syndromes in horses, caseous lymphadenitis in sheep and goats, and sporadically infects other species including cattle and humans.[1] Two biotypes of *C pseudotuberculosis* have been identified on the basis of differences in nitrate reduction, results on restriction endonuclease analysis, and restriction fragment length polymorphism.[2-5] In contrast to ovine and caprine isolates, equine strains are capable of reducing nitrate to nitrite. Natural cross-species infection by the specific biotypes is not known to occur.[6] The 3 forms of disease in horses include limb infection, known as ulcerative lymphangitis; external abscesses; and infection of internal organs. The most common form of *C pseudotuberculosis* infection, characterized by formation of deep abscesses primarily in the pectoral area and ventral abdominal area, is referred to as "pigeon fever" or "dryland distemper."[7-10]

Two major virulence factors have been implicated in the pathogenesis of *C pseudotuberculosis* infections, a cytotoxic surface lipid and **phospholipase D (PLD)** exotoxin.[11,12] The lipid coat appears to facilitate intracellular survival of the organism and abscess formation. A PLD toxin of approximately 31.5 kd is produced by all *C pseudotuberculosis* isolates and may promote spread of the infection through increases in vascular permeability.[13-17] Furthermore, PLD toxin may enhance survival and multiplication of the organism via complement depletion and inhibitory effects on phagocytic cells.[18,19] Complications including abortion, lengthy course of disease, and abscess formation in internal organs have been reported.[20-23] Mortality rates for external abscesses are low, but fatalities resulting from organ involvement occur if diagnosis and appropriate antimicrobial treatment is delayed.[20-23]

The mode of infection remains unproven, but it is speculated that the organism is soil-borne and enters the equine host through skin abrasions as experimentally confirmed for caseous lymphadenitis in sheep.[24,25] Flies and other insects are potential vectors for the disease, as indicated by its peak incidence in late summer and fall and increased incidence in years following heavy winter rainfall that favor insect populations.[20,22,a] Recent studies[26,27] in cattle have implicated *Musca domestica* as a vector for disease in dairy cattle in Israel. Insect vectors for disease in horses have not been determined to date, and attempts to culture the bacteria from flies, ticks, or soil samples from farms with diseased horses have been unsuccessful.[8,20,b] Several authors have observed that the seasonal pattern of ventral midline dermatitis, caused by the feeding habits of the horn fly (*Haematobia irritans*) or biting midge (*Culicoides* sp), coincides with the seasonal incidence of abscesses in horses. This could be the result of either

mechanical transmission of *C pseudotuberculosis* by horn flies or midges or contamination of ventral midline dermatitis lesions from other sources such as soil or other fly species.[10,22]

Although *C pseudotuberculosis* infections in horses have been recognized for several decades, few epidemiologic and immunologic data are available and most discussion is subjective or extrapolative from the disease in sheep and goats. The purpose of the study reported here was to develop and use a sensitive molecular assay for detecting the PLD exotoxin gene of *C pseudotuberculosis* in an attempt to identify insect vectors that may be important in propagation of clinical disease.

Materials and Methods

Horses and fly collection—Five farms that housed 358 horses of variable breed, sex, and age, located in Yolo and Solano Counties of northern California were included in this study. Farms, numbered 1 to 5, housed 30, 150, 8, 146, and 24 horses, respectively. All horses were kept in pasture at least half of the time, and they were monitored for clinical signs of naturally occurring *C pseudotuberculosi*s infection. Farms were visited monthly from May through November 2002, at which time flies were trapped and information and samples from clinically affected horses with *C pseudotuberculosis* infection were collected. Flies were collected from individual horses (clinically affected horses and exposed herd mates) by use of nets and from the environment by use of drift nets and light traps. An attempt was made to collect at least 100 flies from each farm, but this was not always possible as a result of weather conditions or the use of pesticides on horses or in the environment. Flies were placed on dry ice immediately following capture, identified, then placed in a lysis buffer[c] for DNA analysis.

Horses were grouped as clinically affected horses with natural infection (n = 41) and as exposed but not infected horses (317). The diagnosis of *C pseudotuberculosis* infection was established on the basis of clinical appearance of horses, seasonality of disease occurrence, and typical location of lesions on the pectoral area or ventral area of the abdomen. Diagnoses were confirmed by culture of nitrate-positive *C pseudotuberculosis* from the lesions. Diagnosis of internal abscesses caused by *C pseudotuberculosis* was made on the basis of typical clinical and laboratory findings for internal abscesses in combination with a synergistic hemolysis inhibition titer of ≥ 512 or culture of the organism at necropsy.[21,23]

Tissue specimens from 10 clinically affected horses were obtained at the time of surgical drainage of external abscesses. One 3- to 4-mm-diameter biopsy specimen was obtained from the skin overlying the abscess, and tissues were placed immediately into lysis buffer.[c] A biopsy was not performed on herd mates that were exposed to the same environment.

Real-time quantitative polymerase chain reaction assay—A real-time **polymerase chain reaction (PCR)**-based **fluorogenic 5' nuclease assay (TaqMan)** was developed to perform quantitative PCR assays (ie, TaqMan PCR assay). A TaqMan PCR system was designed that targeted the *C pseudotuberculosis* PLD gene (GenBank accession No. L16586). Two primers (CpPLP-98f: 5'-CTACAGCAAATCGGCCAGTCT-3'; and CpPLP-163r: 5'-CGTCATCCACGCCTTGAGT-3') and an internal, fluorescent labeled TaqMan probe (CpPLP-120p: 5'-6-FAM-TGCGATTGCCCACCGCGTTTTA-TAMRA-3') were designed by use of a commercial software program.[d] The TaqMan PCR system was designed after a multiple sequence analysis of ovine and equine *C pseudotuberculosis* isolates to ensure detection of organisms from both species. A closely related but clinically irrelevant isolate, *C ulcerans*, was excluded by designing the TaqMan PCR system for *C pseudotuberculosis* against a sequence stretch with low sequence identity between *C pseudotuberculosis* and *C ulcerans*. The TaqMan PCR system for the detection of the **C pseudotuberculosis PLD gene (CpPld)** is designated as the CpPld TaqMan PCR system.

Fly sample processing and DNA extraction—Collected flies were identified immediately after capture with diagnostic characters described by Loomis[28] and then transferred into lysis buffer[c] for DNA extraction in 96-well, deep-well plates and stored at –20°C until sample processing. Before DNA extraction, two 4-mm-diameter grinding beads[e] were placed in each position of a 96-well, deep-well plate and the flies were homogenized by use of a tissue grinder[f] for 2 minutes at 1,000 strokes/min. After a 30-minute period at 4°C, genomic DNA was extracted from the tissue lysates by use of an automated nucleic acid workstation[g] according to the instructions of the manufacturer. Five microliters of the DNA was used to detect *C pseudotuberculosis* by TaqMan PCR assay.

***C pseudotuberculosis* and *C ulcerans* microbial cultures and DNA preparation**—Lyophilized field isolates of *C pseudotuberculosis* biovar *equi* and biovar *ovis* and *C ulcerans* were grown aerobically for 48 hours on Luria-Bertani broth agar plates and cultured overnight at 37°C. Single colonies were picked from cultures with visible colony growth after 24 hours, resuspended in 200 µL PBS solution, and digested with 20 µL of proteinase K and lysis buffer.[h] The DNA was extracted from the bacteria according to the recommendation of the manufacturer. The DNA was eluted in 200 µL of water, and 5 µL were used for the TaqMan PCR assay.

Real-time TaqMan PCR assay—Each PCR assay contained 400nM of each primer; 80nM of the TaqMan probe; and commercially available PCR mastermix[i] containing 10mM Tris-HCl buffer (pH, 8.3), 50mM KCl, 5mM MgCl$_2$, 2.5mM deoxynucleotide triphosphates, 0.625 U DNA polymerase[j]/reaction, 0.25 U of uracil-N-glycosylase[k]/reaction, and 5 µL of the DNA sample in a final volume of 25 µL. Samples were placed in 96-well plates and amplified in an automated fluorometer.[l] Amplification conditions were 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C.

Statistical analysis—The incidence of disease was defined as the number of affected horses at the farm on the date of fly collection. The relationship between the incidence of disease (%) and PLD gene frequency in fly DNA (percentage of flies with positive results for the PLD gene that were trapped at the farm on that date) was analyzed with the nonparametric Spearman rank correlation method by use of a computer software package.[m] Values of *P* < 0.05 were considered significant.

Results

Analytic specificity and sensitivity of the *C pseudotuberculosis* TaqMan PCR system—Analytic specificity was tested by use of 8 characterized field isolates of *C pseudotuberculosis* and *C ulcerans* (**Table 1**). Use of the TaqMan PCR system resulted in a positive signal for colonies of *C pseudotuberculosis* cultures with detectable growth. The DNA extracted from colonies obtained from *C ulcerans* did not yield a positive signal in TaqMan PCR assay confirming analytic specificity for *C pseudotuberculosis* from ovine and equine origin. Analytic sensitivity was indirectly assessed with a standard curve calculated from dilutions of DNA obtained from cultured *C pseudotubercu-*

Table 1—*Corynebacterium* isolates, culture results, and real-time TaqMan PCR assay results

Sample	Identification No.	Isolate	Species/origin	Culture growth	TaqMan PCR single
1	79-448-12A	*C pseudotuberculosis*	Bovine	Colonies	Strongly positive
2	80-490-9A	*C pseudotuberculosis*	Bovine	Colonies	Strongly positive
3	79-389-11	*C pseudotuberculosis*	Equine	Colonies	Strongly positive
4	80-434-11	*C pseudotuberculosis*	Equine/abscess	Colonies	Strongly positive
5	80-395-12	*C pseudotuberculosis*	Equine/abscess	Colonies	Strongly positive
6	80-379-3	*C pseudotuberculosis*	Ovine/herd infection	Colonies	Strongly positive
7	6127B	*C pseudotuberculosis*	Ovine	Colonies	Strongly positive
8	41-5	*C ulcerans*	–	Colonies	Negative

Real-time TaqMan PCR assay = Real-time polymerase chain reaction-based fluorogenic 5' nuclease assay.

Table 2—Results of inhibition studies

DNA combination	CpPld detection (TaqMan signal)				Gain (+) or loss (-) of single (CT)
	Replicate 1	Replicate 2	Mean	SD	
Fly DNA alone	Neg	Neg	NA	NA	NA
Fly DNA alone	Neg	Neg	NA	NA	NA
Fly DNA alone	Neg	Neg	NA	NA	NA
Fly DNA alone	Neg	Neg	NA	NA	NA
Cp DNA alone	20.33	20.42	20.375	0.06	NA
Cp DNA alone	19.61	19.67	19.64	0.04	NA
Cp DNA alone	22.44	22.6	22.52	0.11	NA
Cp DNA alone	20.65	20.08	20.365	0.40	NA
Fly DNA + Cp DNA	20.65	20.15	20.4	0.35	+ 2.5%
Fly DNA + Cp DNA	19.93	19.51	19.72	0.30	+ 8.0%
Fly DNA + Cp DNA	22.68	22.43	22.555	0.18	+ 3.5%
Fly DNA + Cp DNA	20.25	19.91	20.08	0.24	-28.5%

CpPld = *C pseudotuberculosis* phospholipase D gene. Cp = *C pseudotuberculosis*. CT = Cycle threshold. Neg = Negative. NA = Not applicable.

losis isolates. On the basis of the slope of the standard curve, the amplification efficiency was calculated by use of the formula $E = 10^{-1/slope}$, where E is the percent efficiency and the slope is that of the standard curve. The amplification efficiency of the CpPld TaqMan PCR systems was 97.4%. TaqMan PCR systems with amplification efficiencies > 95% have an analytic sensitivity of 10 molecules/assay as previously shown.[29,30]

PCR assay inhibition by extracted fly DNA—The potential problem of PCR assay inhibition mediated by components of the flies was addressed in a separate experiment in which the DNA extracted from the *C pseudotuberculosis* field isolates was used to spike DNA extracted from flies testing negative for *C pseudotuberculosis*. Whether fly DNA influenced the signal of *C pseudotuberculosis* DNA was assessed by comparing the 2 signals obtained on *C pseudotuberculosis* DNA with or without DNA obtained from flies (**Table 2**). Three of the 4 samples tested had no inhibition, whereas 1 sample had a reduction in the signal by 28.5%, indicating low-grade inhibition of the PCR assay by fly DNA. To address this problem in the DNA extracted from the flies, the DNA was pretested by use of a universal 18S rRNA TaqMan PCR system[n] for the presence or absence of inhibition. For those DNA samples with detectable presence of PCR inhibition, a 1:5 dilution in water eliminated the inhibition and was used for CpPld TaqMan PCR testing.

Incidence of *C pseudotuberculosis* in extracted DNA—Of the 5 farms studied, 2 farms (with a total of 270 horses) had no horses with *C pseudotuberculosis*

infections, whereas the remaining 3 farms (with 188 horses) had diseased horses. Among the latter 3 farms, farm 1 (with 30 resident horses) had a 10% incidence of diseased horses, farm 2 (with 150 resident horses) had a 23% incidence of diseased horses, and on the third farm 4 of 8 resident horses were diseased. Forty clinically affected horses were observed in October 2002 and 1 in May 2002. Of these 41 clinically affected horses, 38 had external abscesses and 3 (7.3%) had both external and internal abscesses. Of 10 skin biopsy specimens collected from affected horses, 5 had positive results for *C pseudotuberculosis* on PCR assay.

A total of 2,621 single flies or pooled flies (4 flies/pool), which were collected either by trapping or netting directly from horses over a 6-month period (May 6, 2002 to November 11, 2002) were analyzed. Fly species tested include horn flies (*Haematobia irritans*), stable flies (*Stomoxys calcitrans*), face flies (*M autumnalis*), house flies (*M domestica*), biting midges (*Culicoides* sp), blackflies (*Simulium* sp), and deer flies (*Tabanus* sp) and a variety of other insects that typically do not feed on horses, including *Drosophila* sp, *Fannia* sp, and the green-bottle calliphorid. The quality of the DNA extracted from flies with negative results for the PLD gene was tested by use of a universal 18S TaqMan PCR system and was not significantly different from DNA tested from flies with positive results for the PLD gene.

Overall, 64 samples tested positive, all on DNA obtained from single flies with an overall incidence of 2.44% (range, 0% to 20%) for *C pseudotuberculosis* on

Table 3—Overview of samples with positive real-time TaqMan PCR assay results

Farm No.	Date	Collection method	Total No. flies tested	No. positive (%)	Species of flies with positive results (No.)
1	10/17/02	From horses	85	2 (2.4)	*Haematobia irritans* (2)
1	10/17/02	Fly trap	57	0 (0.0)	None
2	10/28/03	Fly trap	145	29 (20.0)	*Musca domestica* (22); *Stomoxys calcitrans* (7)
2	10/28/03	From horses	17	0 (0.0)	None
3	10/28/03	Fly trap	166	32 (19.3)	*M domestica* (31); *S calcitrans* (1)
2	11/26/03	Fly trap	320	1 (0.3)	*M domestica*
3	11/26/03	Fly trap	35	0 (0.0)	None

PCR assay. The first flies with positive results for the PLD gene were detected in mid-October on farm 1, at which time 2 of 85 flies collected from horses had positive results (**Table 3**; incidence of 2.4% for *C pseudotuberculosis* on PCR assay; flies collected from 4 horses). None of the 57 flies collected at the same time in traps at that farm had positive results for the PLD gene. Much higher frequencies of flies with positive results for the PLD gene were detected in late October at farms 2 and 3. Of 145 and 166 flies trapped from these respective farms on October 28, 2002, 29 (20.0%) and 32 (19.3%), respectively, had positive results. No flies with positive results for the PLD gene were found in the comparatively modest sample of flies (n = 17) collected from horses on that date.

Notably, high frequencies of positive signals from flies were coincident with high incidence of diseased horses on those farms (ie, 23% at farms 2 and 4 out of 8 [50%] horses at farm 3). One month later, when most horses were recovering from disease (incidence of disease decreased from 23% to 4% at farm 2 and from 50% to 25% at farm 3), significantly fewer flies had positive results for the PLD gene (Table 3). A positive correlation was found between the incidence of horses with disease on the date of sample collection and the percentage of flies that tested positive on that date (Spearman rank correlation coefficient = 0.61).

Fly species with positive results on PCR assay for the PLD gene were *M domestica*, *S calcitrans*, and *H irritans*. None of the other hematophagous or livestock-associated fly species had positive results, although their sample numbers were much smaller. The absence of positive results for any of the species that are not livestock associated suggests that it is the fly-horse interaction itself that led to the presence of *C pseudotuberculosis* in the 3 species of flies with positive results.

Discussion

The incidence of *C pseudotuberculosis* infection in horses varies markedly between years and seasons.[7,23,a] The disease occurs sporadically at endemic farms and can result in epidemics of disease in naïve horses.[8,31,b] On the basis of our observations, the prevalence of disease on endemic farms is estimated at 5% to 10%.[31] Results of an epidemiologic study[32] of 29 infected herds of dairy cattle in Israel revealed a similar prevalence of 5% in sporadically affected herds, with epidemics of infection affecting up to 35% of the herd. A high annu-

al incidence has been reported to follow winters with greater than average rainfall and temperatures.[22] Such an increase in incidence might result from enhanced breeding, hatching, and survival of various insects suspected of mechanically transmitting the bacterium.[8,20,22] Pronounced monthly variations in incidence manifests as a minimum incidence in winter and spring and a maximum incidence in fall, peaking in October and November.[23] Although infection occurs throughout the year, of 637 affected horses at the University of California–Davis Veterinary Medical Teaching Hospital between 1982 and 1994, 82% of affected horses were reported after June 30.[a] The disease is most prevalent in young horses (> 1 and < 5 years of age), but horses of all ages can be affected when the disease is epidemic on nonendemic farms. No breed or sex predilection has been found for disease.[23] Horses kept in summer pasture and horses in increased contact with conspecifics had an increased risk of disease in 1 study.[a] Results of our study indicate that a large variation exists in the incidence of disease among farms (from no affected horses to an incidence of 50%), with a predominance of affected horses in the autumn. All 5 farms we studied had large numbers of flying insects (eg, *M domestica*, *M autumnalis,* and *S calcitrans*) during the study period, and horses at all farms were observed with ventral midline dermatitis, indicative of active populations of horn flies and *Culicoides* spp. For all farms, the proximity to cattle was < 1 mile. Cattle are the principal larval host for *H irritans* and *M autumnalis*.[28]

In our study, the incidence of flies with positive results for the PLD gene increased with an increase in incidence of diseased horses on the farms and was highest in farms with the most clinically affected horses. It is interesting that the incidence of flies with positive results for the PLD gene was highest in October when most of the clinically affected horses were first observed, and then declined by November when most horses were recovering or had recovered from disease. This pattern suggests that flies may be acquiring the bacteria though direct contact with the draining abscesses of diseased horses. However, because of the small number of farms in our study and limited sample collection dates, this evaluation is preliminary and additional data are needed to confirm a relationship between the incidence of disease and incidence of flies carrying the bacteria.

It should be mentioned that the finding of the PLD gene in flies does not provide direct evidence that viable

C pseudotuberculosis organisms are present, although studies[26,27] in cattle did yield live cultures of *C pseudotuberculosis* from flies feeding on infected cattle and from laboratory-raised *M domestica* fed pure cultures of bacteria. Results of previous studies[20,b] in horses, however, were unable to demonstrate viable bacteria from flies or soil samples from farms with affected horses. However, our study is to our knowledge the first report of evidence of *C pseudotuberculosis* (via positive results for the PLD gene) in the biting flies *S calcitrans* and *H irritans*, which have been considered potential vectors of disease in horses for more than 40 years.[20,22,23]

Our findings do not provide information regarding the reservoir of infection, and it is possible that other environmental factors are required for disease transmission. The incubation period for *C pseudotuberculosis* is not known, but it has been estimated at 21 to 28 days on the basis of studies of temporal clustering of affected horses.[33] It appears that for disease to occur, a sufficient density of susceptible horses and a sufficient number of successful contacts between infected and susceptible horses is required. Several years with only sporadically affected horses leads to an increase in the proportion of young susceptible horses. These horses may then become infected with bacteria appearing from 1 or more of several, as yet undetermined, sources (eg, newly introduced affected horse, local or wind-borne soil, migratory insect, or other). Once present, the disease might then be mechanically vectored locally by insects. In subsequent years, it is conceivable that acquired immunity prevents the development of newly diseased horses until the density of susceptible horses is again high enough to support another epidemic. In our study, the identification of 3 potential insect vectors of the bacterium, as well as the season of increased risk of infection, both imply that husbandry measures, including increased hygiene and insect control, should be considered for reducing the probability of infection in at-risk horses. The development and application of the TaqMan PCR assay method provides a sensitive technique to study the pathogenesis of disease in horses.

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[b]Knight HD. *Corynebacterium infections in the horse*. PhD dissertation, University of California, Davis, Calif, 1969.

[c]ABI lysis buffer, cat. No. 4305895, Applied Biosystems, Foster City, Calif.

[d]Primer Express, Applied Biosystems, Foster City, Calif.

[e]SpexCertiprep, Metuchen, NJ.

[f]GenoGrinder2000, SpexCertiprep, Metuchen, NJ.

[g]6700 automated nucleic acid (ANA) workstation, Applied Biosystems, Foster City, Calif.

[h]Qiagen lysis buffer—AL, DNeasy Tissue Kit, Qiagen, Valencia, Calif.

[i]TaqMan Universal PCR Mastermix, Applied Biosystems, Foster City, Calif.

[j]AmpliTaq Gold DNA polymerase, Applied Biosystems, Foster City, Calif.

[k]AmpErase UNG, Applied Biosystems, Foster City, Calif.

[l]ABI PRISM 7700 Sequence Detection System, Applied Biosystems, Foster City, Calif.

[m]SAS JMP, version 5.0.1.2, SAS Institute Inc, Cary, NC.

[n]Assay-on-Demand, cat. No. HS99999901-s1, Applied Biosystems, Foster City, Calif.

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Correction: Effects of feeding large amounts of grain on colonic contents and feces in horses

In "Effects of feeding large amounts of grain on colonic contents and feces in horses," published May 2004, (2004;65:687–694), References 31–52 were numbered incorrectly. The onlineversion at www.avma.org includes the corrected references, which are reprinted here.

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