

Bacterial 16S ribosomal DNA in house dust mite cultures

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Background: Allergen extracts prepared from *Dermatophagoides farinae* contain significantly more endotoxin than *Dermatophagoides pteronyssinus* extracts, and extracts from both mite extracts contain more endotoxin than pollen extracts. Attempts to culture bacteria from mite cultures have failed to establish the sources of the endotoxin.

Objective: To determine the bacterial sources of endotoxin in mite extracts.

Methods: Live mites of both species were obtained from 2 sources, DNA was extracted from the mites, and DNA encoding bacterial 16S ribosomal RNA was amplified by using specific primers. The amount of bacterial DNA in each mite DNA sample was determined by quantitative PCR using an internal standard, and sequence homologies were determined from amplifications performed by using a high-fidelity DNA polymerase.

Results: DNA from *D farinae* appeared to contain between 11-fold and 24-fold more 16S ribosomal gene copies than the genomic DNA from *D pteronyssinus* ($P \leq .003$). Sequence analysis indicated the dominant presence of at least 3 phylogenetic clusters of Bartonella species (*henselae*, *quintana*, *vinsonii*, and *grahamii*), as well as uncharacterized α -proteobacteria, from both *D farinae* and *D pteronyssinus*. In a few clones, sequences from *Escherichia coli*, *Pseudomonas* species, and *Acinetobacter* species were also identified.

Conclusion: House dust mite DNA contains evidence of Bartonella and other Gram-negative species. These Gram-negative species are likely to be the sources of the endotoxin found in mite allergenic extracts. (J Allergy Clin Immunol 2005;116:1296-300.)

Key words: Allergen extracts, house dust mites, endotoxin, lipopolysaccharide, bacteria, allergen immunotherapy

Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria and are a common environmental contaminant in house dust, tap water, milk,

Abbreviations used

TLR: Toll-like receptor

TM: Template mix

and latex gloves. The dramatic clinical consequences of parenteral endotoxin exposure—chills, fever, hypotension, organ failure, death^{1,2}—simulate the symptoms of Gram-negative sepsis, and most parenteral drugs and biologics are required to undergo rigorous testing to assure that they contain no more than a specified quantity of endotoxin, on the basis of dosing instructions.³ For practical reasons, certain biologics, among them allergen extracts, are not required to undergo evaluation for the presence of endotoxin (21CFR 610.13(b)).⁴ At least 3 previous studies have documented the presence of variable quantities of endotoxin in allergen extracts.⁴⁻⁶ In the most recent study, we showed that the endotoxin content of standardized dust mite allergenic extracts appears to be significantly higher in *Dermatophagoides farinae* than in *Dermatophagoides pteronyssinus* extracts by limulus amebocyte lysate gel clot assay. Our attempts to grow bacteria from mite cultures met with inconsistent results.⁷ In this study, we have attempted to detect DNA encoding bacterial 16S ribosomal RNA sequences in extracts prepared from both species of mite. We have used a quantitative PCR method to quantify the DNA, and high-fidelity PCR to identify the bacterial sequences.

METHODS

Sources

Live mites were obtained by overnight courier from 2 sources; washed of growth media, eggs, and feces with sterile water in a stainless steel sieve (45 μ m mesh); and ground in a dounce tissue grinder with a glass pestle. The 2 sources are the research laboratory of one of the authors (L. G. A.) and a commercial source supplier of mites for some US manufacturers (Biopol, Spokane, Wash). For both

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^dThe Code of Federal Regulations is the codification of the general and permanent rules published in the Federal Register by the executive departments and agencies of the federal government. It is divided into 50 titles that represent broad areas subject to federal regulation. The Code of Federal Regulations may be accessed at <http://www.gpoaccess.gov/cfr/>.

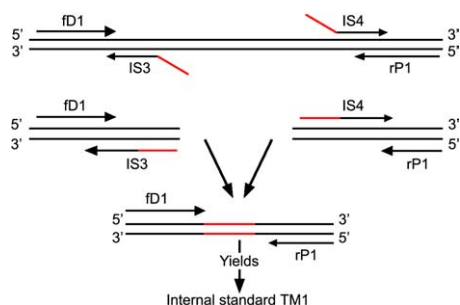


FIG 1. Synthesis of the TM1 internal standard. Equal parts of 2 forward primer pairs, fD1 and IS4, and 2 reverse primer pairs, rP1 and IS3, were mixed with template DNA from *C diphtheriae* strain c7 and amplified. Complementary regions of IS3 and IS4 are shown in red. TM2 was produced similarly by using fD2 instead of fD1 (not shown).

sources, the growth conditions and media for the 2 mite species are identical.

DNA extraction from mites

DNA was extracted with DNAzol (Invitrogen, Chicago, Ill), the mass of extracted DNA was estimated by absorbance at 260 nm, and the purity was estimated by the ratio of absorbances at 260 nm and 280 nm.

PCR primer sequences

The 16S ribosomal RNA sequences were detected and characterized by PCR using sequences previously described.⁸ The fD1 and rP1 primers are designed to detect most prokaryotic sequences; the fD2 primer—different in 1 position—is more specific for enteric bacteria. Primers fD1 and fD2 contain *EcoRI* and *SalI* sites; rP1 contains *HindIII*, *BamHI*, and *XmaI* sites; and these cloning site sequences are in lower-case. IS3 is a reverse primer that contains a 20-base region homologous to an internal sequence in the 16S ribosomal code, and the IS4 primer recognizes another internal 16S ribosomal sequence separated from IS3 sequence by about 940 bases. Because the IS3 and IS4 primers also contain a common complementary region, they generate a shorter DNA product that can be used as an internal standard for the fD1/rP1 or fD2/rP1 amplifications.

The complementary regions are in italics.

Reverse primer rP1:

cccgatccaagcttACGGTTACCTTGTTACGACTT

Forward primer fD1:

ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG

Forward primer fD2:

ccgaattcgtcgacaacAGAGTTTGATCATGGCTCAG

Internal sequences:

IS3: TGTGTGAAGCCATTACCCACCAACAAGCTG

IS4 (3' to 5'):

AACATCGTACACTTCGGGTAATGGGGTGGT

Internal standards synthesis

Template mix (TM) 1 contained equal parts of 2 forward primer pairs, fD1 and IS4, and 2 reverse primer pairs, rP1 and IS3. TM2 also contained equal parts of 2 forward primer pairs, fD2 and IS4, and 2 reverse primer pairs, rP1 and IS3. Amplification mixtures contained Platinum PCR Supermix (Invitrogen), the indicated primers, and template DNA from *Corynebacterium diphtheriae* strain c7,

TABLE I. Yields and purity of DNA prepared from mites obtained from 2 sources

	Source	DNA yield (mg)	DNA yield (μg DNA/mg mite)	A ₂₆₀ /A ₂₈₀
<i>D farinae</i>	Arlan	7.13	103.4	1.58
	Biopol	1.21	13.1	1.43
<i>D pteronyssinus</i>	Arlan	1.98	29.6	1.76
	Biopol	2.26	34.3	1.45

and were amplified in a Strategene Robocycler by using the following conditions: 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for 10 minutes. The internal standards were purified, digested with *EcoRI* and *HindIII*, and ligated into pUC 19. The plasmids containing the inserts were grown in large quantity, purified, quantified carefully by absorbance at 260 nm, and stored in the presence of 5 μg/mL glycogen (Roche, Basel, Switzerland) at −20°C (Fig 1).

Quantification of 16S ribosomal sequences in mite DNA

To estimate the amount of 16S ribosomal RNA in the DNA extracted from the mites, 16S ribosomal sequences were amplified from varying amounts of mite DNA in the presence of the internal standards TM1 or TM2, present at either 10³ or 1.5 × 10³ copies per tube.⁹ PCR conditions were as follows: Platinum PCR Supermix (Invitrogen), forward and reverse primers (fD1 or fD2 and rP1), genomic DNA, internal standard TM1 or TM2 (1.62 pmol/L). Amplification was performed in an Applied Biosystems (Foster City, Calif) GeneAmp PCR System 9700: 94°C for 2 minutes, 10 cycles of 94°C for 10 seconds, 55°C for 30 seconds, and 68°C for 2 minutes; and 25 cycles of 94°C for 10 seconds, 55°C for 30 seconds, and 68°C for 2 minutes, with 20 seconds added for each consecutive cycle.¹⁰

The amplification products were resolved on a 2% agarose gel in Tris acetate EDTA buffer containing 5 μg/mL ethidium bromide, and the gel images were captured on an Alpha Innotech (San Leandro, Calif) Imager ChemilImager 4400 by using Alpha Ease version 5.1 software. Band densities were determined by using Scion Image for Windows (version beta 4.0.2; Scion Corporation, Frederick, Md). Amplification product band densities were plotted against the log mass concentration of the mite DNA, a best fit line was determined by linear regression, and the mite DNA mass with the same band density as that of the internal standard was determined by interpolation. Only best fit lines with an *r*² greater than 0.9 by Pearson analysis were accepted. 16S ribosomal sequence quantifications were analyzed by paired *t* tests. Significance was evaluated by paired *t* tests of log mass concentration values.

A sample calculation follows. In 1 experiment, extracted DNA from *D farinae* was amplified from the following starting amounts: 10, 3.3, 1.1, and 0.37 ng DNA. After amplification and agarose electrophoresis in the presence of ethidium bromide, the normalized densities of the resolved bands were 15.99, 14.03, 3.59, and 0.94 arbitrary units, respectively. A log-linear regression analysis for these data yielded the regression curve $y = 0.08x - 0.40$, where $y = \log(\text{DNA})$ and $x = \text{band density}$ ($r^2 = 0.92$). In the same experiment, the mean density for 4 separate samples of TM1, each containing 10³ copies, was 5.72 ± 0.76 . Solving the regression equation for the band density 5.72 yields a DNA mass of 1.14 ng. Hence, the densities of the amplification products from *D farinae* DNA and from TM1 indicated the presence of 10³ copies of bacterial DNA in 1.14 ng of extracted mite DNA, or 877 copies/ng. In the same experiment, the

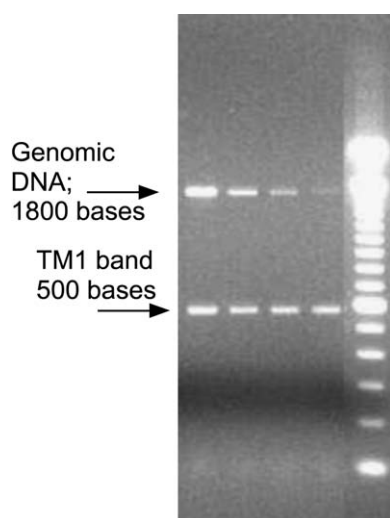


FIG 2. Gel electrophoresis of a typical coamplification of mite DNA (30, 10, 3.3, and 1.1 ng/tube) with TM1 (1000 copies/tube). The amplification products were resolved on a 2% agarose gel with ethidium bromide, the band densities were measured, and the bacterial DNA content of each mite DNA extract (in copies/ng genomic DNA) was determined by interpolation on a log mass linear regression curve (see Table II).

D pteronyssinus DNA amplifications indicated the presence of 53 copies of bacterial DNA/ng of extracted mite DNA.

Sequencing

DNA was amplified in Applied Biosystems Gene Amp PCR System 9700 as detailed by using high-fidelity PCR Accuprime pfx Supermix (Invitrogen). The amplification products (1800 bp) were digested with *Bam*HI and *Sal*I and ligated into pUC19. Ten random clones of each primer pair from each mite species were selected for sequencing (MWG Biotech, High Point, NC). Sequence similarities were analyzed by Basic Local Alignment Search Tool (BLAST).¹¹ Phylogenetic relationships among the sequences were assessed after alignment by using GCG-Lite+ (National Institutes of Health, Bethesda, Md) with the fastDNAmI program and bootstrapped 100 times.¹²

RESULTS

By using standard techniques, we isolated DNA from washed mites obtained from 2 source suppliers (Table I). Amplification of the mite DNA from both species using both primer sets directed at 16S ribosomal RNA (fD1/rP1 and fD2/rP1) yielded DNA of approximately 1800 bases, as expected.⁸ To quantify the bacterial DNA in the mite preparations, we produced internal standards TM1 and TM2, which contain the same 5' and 3' primer sites separated by a shorter interval than in the native construct (Fig 1). When we coamplified mite genomic DNA with the internal standards TM1 or TM2, the DNA from *D farinae* appeared to contain between 11-fold and 24-fold more 16S ribosomal gene copies than the genomic DNA from *D pteronyssinus* (Fig 2 and Table II). These differences were significant by paired *t* test ($P \leq .003$).

TABLE II. Calculated copy numbers (copies/ng mite genomic DNA) of 16S ribosomal DNA for DNA extracted from both mite species from 2 sources, on the basis of coamplification using fD1 and rP1 primers concurrently with the TM1 internal standard, or fD2 and rP1 primers concurrently with the TM2 internal standard (see Fig 2)*

		Source A		Source B	
		Dp	Df	Dp	Df
fD1/rP1	Mean (copies/ng DNA)	100	1108	94	1300
	SD (log)	0.10	0.09	0.18	0.17
	<i>P</i> value	.01		<.001	
	N	3	3	4	4
fD2/rP1	Mean (copies/ng DNA)	71	1085	45	1060
	SD (log)	0.29	0.45	0.29	0.40
	<i>P</i> value	.002		.003	
	N	4	4	3	3

*In materials obtained from both sources, DNA extracted from *D farinae* contained a significantly greater number of copies of DNA encoding bacterial 16S ribosomal RNA. *N*, Replicate analyses performed on the DNA preparations. *P* values were calculated by paired *t* tests of log mass concentration values.

The PCR sequence data with both fD1 and fD2 primers (Tables III and IV) yielded sequences for the 16S ribosomal DNA from various *Bartonella* species (*henselae*, *quintana*, *vinsonii*, and *grahamii*), uncharacterized α -proteobacteria, and uncharacterized endosymbionts from ticks (*Ixodes scapularis*), tubeworms (*Vestimentiferan* species), and other mites (*Metaseiulus occidentalis*, *Brevipalpus phoenicis*, and *Aspidiotus nerii*). In several clones amplified with the fD2 primer, we obtained sequences from *Escherichia coli*, *Pseudomonas* species, and *Acinetobacter* species (Table IV). At least 3 phylogenetic clusters of *Bartonella* organisms were identified (not shown).

DISCUSSION

Our previous work documented the presence of endotoxins⁶ in allergen extracts. In this study, we attribute the elevated levels of endotoxin in house dust mite extracts to the presence of several Gram-negative organisms, predominantly *Bartonella*. Extracted DNA from *D farinae* and *D pteronyssinus* contain bacterial DNA encoding 16S ribosomal RNA. The 16S ribosomal sequences identified were closely homologous to published sequences from *Bartonella* species, as well as several enteric bacteria, any of which could be the source of the endotoxin detected.¹³ Just as *D farinae* allergen extracts contain more endotoxin than *D pteronyssinus* extracts,⁶ the DNA obtained from *D farinae* contains a significantly greater number of copies of the bacterial sequences than does the DNA extracted from *D pteronyssinus* (Table II).

Bartonella species are facultative intracellular Gram-negative rods associated with at least 1 zoonotic disease

TABLE III. 16S ribosomal sequences from high-fidelity PCR of mite DNA using primers fD1 and rP1*

	Source	Submitted sequences	Bartonella	α -Proteobacterium	Uncharacterized
<i>D. farinae</i>	Arlian	10	10		
	Biopol	4	2	1	1
<i>D. pteronyssinus</i>	Arlian	2	2		
	Biopol	6	6		

*Forty clones were selected at random (10 from each group), but sequence was obtained from the 22 clones indicated. Homologies were determined by BLAST search.¹¹

TABLE IV. 16S ribosomal sequences from high-fidelity PCR of mite DNA using primers fD2 and rP1*

	Source	Submitted sequences	Bartonella	Gram-negative enterics	Uncharacterized
<i>D. farinae</i>	Arlian	8	4	3	1
	Biopol	7	2	5	
<i>D. pteronyssinus</i>	Arlian	9	6	3	
	Biopol	7	5	2	

*Forty clones were selected at random (10 from each group), but sequence was obtained from the 31 clones indicated. Homologies were determined by BLAST search.¹¹

(cat scratch disease), several louse-borne illnesses (Oroya fever, verruga peruana, trench fever, urban trench fever), and recently described diseases of uncertain transmission (bacillary angiomatosis, bacillary peliosis, and culture-negative endocarditis).^{14,15} In addition to the body louse, *Bartonella* have been detected in fleas^{16,17} and ticks.¹⁸ In this report, we provide evidence that *Bartonella* organisms are harbored by house dust mites as well. There is no evidence of disease transmission by dust mites, although other mites have been implicated as vectors in rickettsial diseases in human beings (rickettsialpox and scrub typhus), in addition to disease transmission among plants and animals.

Whether these organisms exist in the mite cultures as endosymbionts or stable contaminants cannot be determined from the current study. Although *Bartonella* have been identified in several arthropod species, vertical transmission data support endosymbiosis only in Hippoboscidae flies.¹⁹ If *Bartonella* bacteria exist as true endosymbionts in *Dermatophagoides* mites as well, eradication from existing cultures may be impossible.

House dust mite extracts are prepared by washing and grinding the cultured mites and extracting them in an aqueous neutral buffer. After extraction, the extracts are allowed to sediment, are clarified through a series of coarse filtration steps, and then are filter-sterilized. The final buffer solutions for US extracts all contain glycerol (50% vol/vol); most contain phenol 0.4% (wt/vol) as well. *Bartonella* are fastidious organisms, 0.6 μ m $\times$ 1.0 μ m, and would be expected to be quantitatively removed by sterile filtration (0.2- μ m or 0.45- μ m filters)²⁰ and destroyed by phenol. There have been no reports of iatrogenic *Bartonella* infection.

Like other Gram-negative organisms, *Bartonella* species contain abundant lipopolysaccharide, but *Bartonella*-derived lipopolysaccharide has some unusual functional and structural features. Although strongly

reactive by limulus amoebocyte lysate gel clot assay and similar to other endotoxins in the induction of several inflammatory responses, *Bartonella* lipopolysaccharide fails to induce TNF- α or vascular collapse in rats.¹³ Furthermore, *Bartonella* lipopolysaccharide has sharply reduced interactions with Toll-like receptor (TLR) 4, and none with TLR2.²¹ Recent attention on the effects of lipopolysaccharides on atopic responses has been focused on TLR4 polymorphisms. Individuals with decreased TLR4 function appear to have increased likelihood of T_H2 responses and atopy.²² Thus, although one might expect the presence of endotoxin in an allergen immunotherapy mixture to enhance T_H1 responsiveness to the allergens,²³ the effects of lipopolysaccharides derived from *Bartonella* on the efficacy of allergen immunotherapy are uncertain.

Questions for further study raised by these observations include the following:

- The data from the 2 independent sources of mites in this study were nearly identical. Would wild mites or cultured mites from other sources yield the same results?
- What is the biological relationship between the bacteria and the mites? Why is there a quantitative difference between the bacterial load of the 2 mite species?
- Is there a difference in the efficacy of *D. farinae* and *D. pteronyssinus* allergenic extracts used in allergen immunotherapy? If so, what role does the greater amount of endotoxin in *D. farinae* extracts play in this difference?

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