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Detecting and quantifying mites in domestic dust: A novel application for real-time PCR

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1. Introduction

Real-time quantitative PCR (qPCR) is a widely used method to quantify the abundance of organism or gene expression in the environment. QPCR based analyses combine classical DNA amplification by PCR with real time counting of amplicons during each cycle of PCR amplification. Target specificity of qPCR assays is determined by the design of the primers and in some cases internal probes. Indeed, qPCR analyses have been developed to quantify species belonging to fungi and protista kingdoms, and are currently used in human environment investigations of allergies to assess allergen exposure (Haugland et al., 2004; Kaarakainen et al., 2011; Vesper et al., 2007b). The advantage of the qPCR approach is its ability to quantify numerous individual species in a complex mixed community with a single tool. QPCR to detect fungi was used to investigate the relationship between mold exposure and asthma (Reponen et al., 2012; Vesper et al., 2007a) but has not yet been developed for house dust mite quantification, although there is good evidence suggesting a causal relationship between mite exposure and allergic disorders such as asthma (Cole Johnson et al., 2004; Lodge et al., 2011; Platts-Mills et al., 1997). Mites found in house dust causing the development of IgE antibody responses are mainly house dust mites (from the Pyroglyphidae family, *e.g. Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Dermatophagoides microceras* and *Euroglyphus maynei*), storage mites (*e.g. Lepidoglyphus destructor*, *Acarus siro* and *Tyrophagus putrescentiae*) and other species such as *Blomia tropicalis* (Colloff and Spieksma, 1992; Platts-Mills et al., 1992). The ELISA was the most commonly used method for investigating allergens from these mites in the environment (Dunn et al., 2008; Krizkova-Kudlikova et al., 2007). In our study, we propose to broaden the use of qPCR assays to quantify mites in the environment. Primers and probes were designed to detect and measure amounts of nucleic acids from *D. pteronyssinus*, *D. farinae, Dermatophagoides species* and, less specifically, from several mites present in domestic dust. Analytical characteristics (specificity, linear dynamic range, limit of detection and efficiency) were determined to validate each qPCR assay. QPCR was tested on 19 dust samples for mattresses and the results were compared to the results of Der p I quantification (ELISA) and to the results of guanine semi-quantification test (ACAREX® test).

2. Material and methods

2.1. Tested strains

D. pteronyssinus, *D. farinae, L. destructor, Glycyphagus domesticus* and *A.* siro (Mite collection, Laboratory of Parasitology, Faculty of Pharmacy, University of Basque Country, Vitoria, Spain) were used to test specificity. The strains were cultured on a 1:1 (wt:wt) autoclaved mixture of commercial mouse meal and dried yeast powder according to Cardona et al. (2004).

Individuals of *Ephestia kuehniella* were collected in a house using glue traps.

Fungal species used were those currently found in indoor dust samples. Stains are deposited in the fungal BCCM/IHEM collection (Scientific Institute of Public Health, Brussels, Belgium): *Aspergillus versicolor* (BCCM/IHEM 22975), *Cladosporium sphaerospermum* (BCCM/IHEM 18883), *Penicillium chrysogenum* (BCCM/IHEM 22667), *Aspergillus*

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fumigatus (BCCM/IHEM 22670), *Alternaria alternata* (BCCM/IHEM 22669), *Eurotium amstelodami* (BCCM/IHEM 16286) and *Wallemia sebi* (BCCM/IHEM 16284). Fungi were cultured onto Sabouraud or Malt Agar media (Beckton Dikinson, New Jersey, USA) at 20 or 30 °C for two weeks. Spores were removed using sterile distilled water and were recovered by centrifugation (10 min at 6000 ×*g*).

2.2. DNA isolation

Two hundred microliters of DNA-free water containing almost 400 mites, 3 individuals of *E. kuehniella* or 10 mm^2 fungal suspension was placed into 2.0 ml conical-bottom screw-cap tubes (MagNA Lyser Green Beads, Rochediagnostics GmbH, Germany) containing 1.4 mm ceramic beads and 400 µl of lysis buffer from the NucleoSpin Plant II kit (Macherey-Nagel, Germany). The tubes were shaken in a MagNA Lyser Instrument (Roche Applied Science®, Germany) three times for ten seconds at the maximum speed, with one cooling minute in the cooling block. The tubes were then heated in a boiling water bath for 10 min. The samples were placed on ice for 10 min, followed by centrifugation for 2 min at 6000 ×*g*. DNA was then purified with NucleoSpin Plant II kit according to the manufacturer's instructions. The DNA concentration of each extract was determined by a dosage program (160 nm) with a spectrophotometer (ND-1000 Nanodrop, Thermo Fisher Scientific, USA).

2.3. QPCR

PCR reactions contain 10 µl two-fold concentrated TaqMan® Gene Expression Master Mix (Applied Biosystems, USA), reverse and forward primers (final concentration 1 µM each) (Sigma-Aldrich, USA), a hydrolysis probe (final concentration 200 nM) (MWG Eurofins Operon, Germany), 5 µl of DNA sample and nuclease-free water to complete the 20 µl total volume of reaction mixture. Plates were centrifuged (one min at 1000 ×*g*) and PCR reactions were run and monitored using Applied Biosystems 7500 FAST Real-Time PCR System and 7500 Software v2.0.5. Sterile DNA-free water was extracted with samples and used as negative controls in each series of amplification. Thermal cycles consisted of 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

2.4. Design of probes and primers and analytical specificity

Eighteen rRNA sequences of the main mite species isolated indoors were retrieved from the GenBank database and were aligned using BioEdit Software (Tom Hall Ibis Biosciences, California) to choose the most appropriate areas for primer design. Aligned sequences were those of *D. pteronyssinus* (accession numbers: EU152579; DQ025512; DQ025511), *D. farinae* (GQ864309; EU152578; GQ864309), *Dermatophagoides evansi* (EU152577), *A. siro* (AF022023; EU152495), *L. destructor* (EF203771), and *T. putrescentiae* (DQ025510). Primers and probes were designed using Primer Express® 3.0 for Real-Time PCR software (Applied Biosystems). Parameters used to design primers and probes were chosen to ensure optimal conditions for qPCR. Indeed, the minimum and maximum melting temperatures (T_m) permitted for primers were 58 and 60 °C respectively. The minimum and maximum percentages of C and G contained by either primer were 30 and 80% respectively, with a maximum of two residues on the 3′ end required to be a G or C. The optimal length allowed for each primer was 20 bases (9 bases minimum and 40 bases maximum). Minimum and maximum T_m allowed for probes were 68 and 80 °C respectively. The minimum and maximum percentages of G and C contained by the probes were 30 and 80% respectively for a length range of 13 to 30 bases. No more 3 G repeats were required in probe. The amplified region length permitted ranged from 50 to 150 bases.

Analytical specificity, which refers to the qPCR assay detecting the appropriate organism rather than the other, was tested against seven fungal and five mite DNA extracts at 1 pg/µl (Sections 2.1 and 2.2).

2.5. QPCR linear dynamic range, limit of detection and efficiency

To generate calibration curves, DNA extracts were adjusted at 100 pg/µl in the NucleoSpin Plant II kit elution buffer, and were then serially (1:10) diluted, resulting in a six point calibration curve range of 100 to 0.001 pg/µl. QPCR analysis was repeated three times for each concentration. For each qPCR assay, the calibration curve allowed us to calculate the linear dynamic range, the limit of detection (LOD) and PCR amplification efficiency. The linear dynamic range is the concentration range over which the quantification cycle (Cq) and concentration remain directly proportional to each other. Range interval and correlation coefficient (r^2 value) were then reported. The LOD was the lowest concentration at which DNA was detected in all three samples. PCR amplification efficiency was established as the slope of the log–linear portion of the calibration curve: PCR efficiency $= 10^{-1/\text{slope}} - 1$.

2.6. Applying qPCR assays on dust from mattresses

Dust from 19 mattresses belonging to people with allergic rhinitis or asthma was collected using nylon sampling socks that were designed to fit on a vacuum cleaner. The whole area of the mattress from which the sample was taken was cleaned for 15 min. The socks were then removed from the vacuum cleaner and closed.

The ACAREX® test (Dyn'R, France) was used according to the manufacturer's instructions directly after dust collection. A colorimetric scale can determine the amount of guanine in dust according to four levels: none, low, medium and high.

In the laboratory, dust samples were filtered (hole of 1 mm in diameter) and stored at −20 °C. To prepare the DNA extracts as described in Section 2.2, 15 mg of dust was mixed with 200 µl of DNA-free water. QPCR assays were performed for each target three times on different days and by different technicians, with the aim of calculating reproducibility expressed by the coefficient of variation from the three points after conversion in pg/µl using calibration curves.

Concentrations of Der p I in dust were determined by ELISA assays (Der p I Assay, Citeq Biologics, The Netherlands) according to the manufacturer's instructions. Results were grouped into three categories according to calibration points: less than 0.05 µg Der p I per gram of dust, between 0.05 and 0.4 µg/g and more than 0.4 µg/g.

Table 1
Primers and probes[a] list.

Dermatophagoides pteronyssinus		
Fwd	Dpter F1	TGTTGTGGTTAAAAAGCTCGTAGTTG
Rev	DpterR1	ATGCGATAATCTGCTCAGTATGACA
Probe	DpterP1	CAGCTCATGTATGGCGGTCCACCTG
Dermatophagoides farinae		
Fwd	DfariF1	TGTTGTGGTTAAAAAGCTCGTAGTTG
Rev	DfariR1	ATGCGATATTCTGCTCAGCATGACA
Probe	DfariP1	CAGCTCATGCATGGTGGTCCACTTG
Dermatophagoides spp.		
Fwd	DermaF1	CCTCGTGGTGGAGTGCATT
Rev	DermaR1	TCAGCTGCAGTTATCCAGAGTCA
Probe	DermaP1	ATTAGACCAAGACCAAAGGTGGCAACACC
Mites		
Fwd	MisppF1	GAGGTTCGAAGGCGATCAGA
Rev	MisppR1	TTGCTGGTTGGCATCGTTTA
Probe	MisppP1	ACCGCCCTAGTTCTA

Fwd: forward primer; Rev: Reverse primer.
[a] Labeled with FAM reporter and TAMRA quencher.

Table 2
Mean of threshold cycles (standard deviation) for the specificity study of qPCR.

Species extracts	DpterF1/R1/P1	DfariF1/R1/P1	DermaF1/R1/P1	MisppF1/R1/P1
Dermatophagoides pteronyssinus	35.10 (0.53)	Und.	36.84 (0.85)	37.59 (0.36)
Dermatophagoides farinae	Und.	35.35 (0.42)	36.15 (0.81)	37.31 (0.55)
Lepidoglyphus destructor	Und.	Und.	Und.	36.79 (0.55)
Glycyphagus domesticus	Und.	Und.	Und.	36.28 (0.98)
Acarus siro	Und.	Und.	Und.	39.99 (1.64)

Und. (undetermined): no amplification curve was obtained after 45 cycles of PCR.

3. Results

3.1. Design of primers/probes and analytical specificity

The alignment of mite sequences enabled us to choose areas for primer and probe designs. Primer and probe sequences are listed in Table 1. DpterF1/R1/P1 was designed specifically to amplify *D. pteronyssinus*; DfariF1/R1/P1 specifically to amplify *D. farinae*; and DermaF1/R1/P1 to amplify *D. pteronyssinus* and *D. farinae*. MisppF1/R1/P1 was designed in a conserved area in sequences, so as to detect several genera of mites.

Analytical specificity was first validated using BLAST searches, and then by qPCR assays against mites, *E. kuehniella* and fungal extracts. No fungal or insects DNA extracts were detected in qPCR assays using the four primers/probes designed. Mite extracts were amplified as reported in Table 2, showing a good analytical specificity: only the target species were detected. For MisppF1/R1/P1, we found 3.7 cycles of difference between *G. domesticus* and *A. siro* amplification, all tested at 1 pgDNA/µl. This suggests that there were differences in PCR efficiency or that target repetition was not the same for each of the two species.

3.2. Analytical characteristics of qPCR assays

Linear dynamic ranges and LOD of qPCRs were calculated using calibration curves of targeted organisms (Table 3). Linear dynamic ranges were between 100 pg/µl and 1 or 0.1 pg/µl depending on qPCR assays. Correlation coefficients were up to 0.99 for DpterF1/R1/P1, DfariF1/R1/P1 and DermaF1/R1/P1, showing a good linearity between logarithms for DNA concentration and Cq. A weaker correlation coefficient was obtained with the MisppF1/R1/P1 assay performed with the *D. pteronyssinus* calibration curve. The limit of detection ranged from 10^{-3} to 10^{-5} equivalent mites per microliter of extract.

Coefficients of variation for reproducibility, established on dust sample extracts, were higher for MisppF1/R1/P1 than for the three other qPCR assays. Coefficients of variation higher than 0.8 are not acceptable in qPCR because they represent a very high dispersion of Cq values for the same DNA extract.

3.3. Applying qPCR to mattress dust

QPCR assays on the 19 dust extracts were performed using DermaF1/R1/P1 primers and probes. Quantities of *Dermatophagoïdes* ranged from 1 to 532 mites per gram of dust, with a mean of 78 mites per gram (standard deviation (SD) = 136).

Acarex® test results showed that the amount of contamination caused by mites was low in five mattresses, medium in ten mattresses and high in four mattresses. There was no correlation between the qPCR and Acarex® test results in mattress dust (Fig. 1).

ELISA Der p I results were correlated with the quantities of *Dermatophagoides* determined by qPCR assays ($r^2 = 0.55$) (Fig. 2). A mean quantity of four *Dermatophagoides*/g of dust (SD = 2) were determined by qPCR when Der p I was found to be less than 0.05 µg/g of dust, 13 *Dermatophagoïdes*/g (SD = 8) when Der p I in dust was 0.05 to 0.4 µg/g and 140 *Dermatophagoides*/g (SD = 168) when Der p I was more than 0.4 µg/g.

4. Discussion

We proposed and validated a method to quantify house dust mites using qPCR. Four primers/probes were designated to target *D. pteronyssinus* (DpterF1/R1/P1), *D. farinae* (DfariF1/R1/P1), *Dermatophagoides species* (DermaF1/R1/P1) and the main mites present in domestic dust (MisppF1/R1/P1). The qPCR experiments were validated following the MIQE guidelines (Bustin et al., 2009). The specificity of targets was validated by tools for sequence aligning and by direct experimental assays. The linear dynamic ranges covered three or four orders of magnitude and was range to 3 or 2 $\log_{10}$. Ideally, the linear dynamic range should be 5 $\log_{10}$ but our assays were restricted by the limit of detection. We should thus have tested more concentrate DNA extracts to reach 5 $\log_{10}$. The limit of detection for the qPCR experiments mainly ranged from 1 to 0.1 pg/µl, allowing us to detect 13 to 1 mites per gram of dust. This limit of detection is equivalent to those of the ELISA Der p I method (Lind, 1986) and is higher to those of the ELISA allowing detection of *A. siro* in grain (Krizkova-Kudlikova et al., 2007).

For the last twenty years, three methods have been used in environmental studies to estimate exposure to mites: mite counts, assays for specific mite allergens by ELISA, and measurements of guanine, the major nitrogenous excretory product of arachnids (Platts-Mills et al., 1992). These three methods are correlated with each other (Hallas et al., 1993; Hoyet et al., 1991; Lind, 1986; Van Bronswijk et al., 1989). Mite counts by microscopic analysis allow to specify the founding species and demonstrate a good correlation with group I allergen quantification (Platts-Mills et al., 1992). However, determining species requires expert knowledge and this method

Table 3
Performance of qPCR assays to detect specific mite strains extracted.

Targets		Linear dynamic range		Limit of detection		PCR efficiency in %	CV_{rep}
Primers/probes	DNA extract tested	Interval	r^2	In pg/µl	In mites/µl		
DpterF1/R1/P1	*D. pteronyssinus*	100 to 0.1 pg/µl	0.998	1	10^{-3}	74	0.52
Dfari F1/R1/P1	*D. farinae*	100 to 1 pg/µl	0.990	0.1	10^{-4}	73	0.41
DermaF1/R1/P1	*D. pteronyssinus*	100 to 1 pg/µl	0.995	1	10^{-3}	70	0.43
DermaF1/R1/P1	*D. farinae*	100 to 1 pg/µl	0.996	0.1	10^{-4}	79	0.40
MisppF1/R1/P1	*D. pteronyssinus*	100 to 1 pg/µl	0.974	0.1	10^{-4}	63	0.59
MisppF1/R1/P1	*D. farinae*	100 to 1 pg/µl	0.997	0.1	10^{-4}	78	0.77
MisppF1/R1/P1	*A siro*	100 to 0.1 pg/µl	0.981	1	10^{-3}	75	0.82
MisppF1/R1/P1	*L. destructor*	100 to 0.1 pg/µl	0.997	1	10^{-3}	85	0.86
MisppF1/R1/P1	*G. domesticus*	100 to 1 pg/µl	0.999	0.01	10^{-5}	81	0.90

CV_{rep}: coefficient of variation for reproducibility.

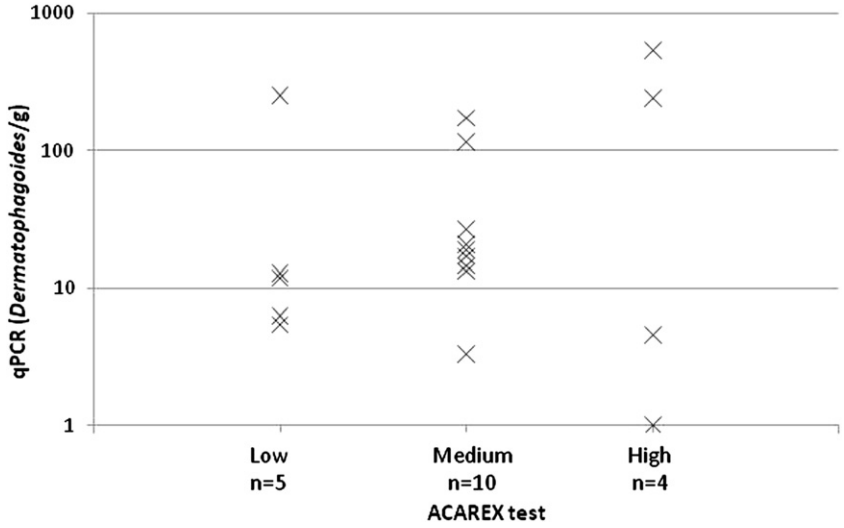


Fig. 1. Correlation between qPCR assays (DermaF1/R1/P1) and ACAREX® tests in mattress dust (n = 19).

takes too long to be applicable to a large number of samples. Guanine production of mites is also correlated with allergen Der p I and with mite counts (Koren et al., 2000). Quantification is limited by the amounts of guanine not originate to house dust mites (Hallas et al., 1993). The ELISA was the most commonly used method for investigating the human environment with species-specific monoclonal antibodies to bind group I (Der p I and Der f I) allergens. Measurements were usually performed on dust from floor and mattresses (Brunekreef et al., 2002; Gent et al., 2012). The generalized use of molecular biology has made it possible to propose new methods for quantifying mites in the environment.

The results of detection and quantification of *Dermatophagoides* species in dust by qPCR correlated with ELISA assays for Der p I quantification, as there is a correlation between mite counts and Der p I quantification (Lind, 1986). Quantities of dust necessary for assays are different for qPCR and ELISA tests: 100 mg of dust was needed to perform the ELISA whereas qPCR experiments were performed with 15 mg of dust. In qPCR experiments, a single dust extract allows us to quantify fungi and bacteria as well as house dust mites. The human environment is a complex biota in which these organisms interact (Naegele et al., 2012). The study of these interactions may be facilitated by a single method to quantify all organisms.

We did not show a clear relationship between the qPCR assay and ACAREX® test results. However, studies comparing ACAREX® and ELISA tests showed that ACAREX® was especially accurate in detecting negative results (level 0: none) and highly positive results (levels 2 and 3: medium and high) (Haouichat et al., 2000; Ridout et al., 1993). The ACAREX® test is simple to use in the domestic environment. It is used to identify sources of mites in houses. This colorimetric test has educational properties that can help involve the patient in finding remedies, but is not used to quantify allergen exposure.

Variation coefficients for reproducibility were better for assays specific to one or two species (DpterF1/R1/P1, DfariF1/R1/P1 and DermaF1/R1/P1) than those detecting a large range of species (MisppF1/R1/P1). Furthermore, the correlation coefficient of calibration curves and PCR efficiency were also lower for MisppF1/R1/P1. We thus recommend using the DpterF1/R1/P1, DfariF1/R1/P1 and DermaF1/R1/P1 specific targets for assessing environmental exposure.

5. Conclusions

New developments in molecular biology have enabled us to quantify organisms in the human environment quickly. QPCR is thus used in epidemiological studies on asthma and allergy to quantify fungi.

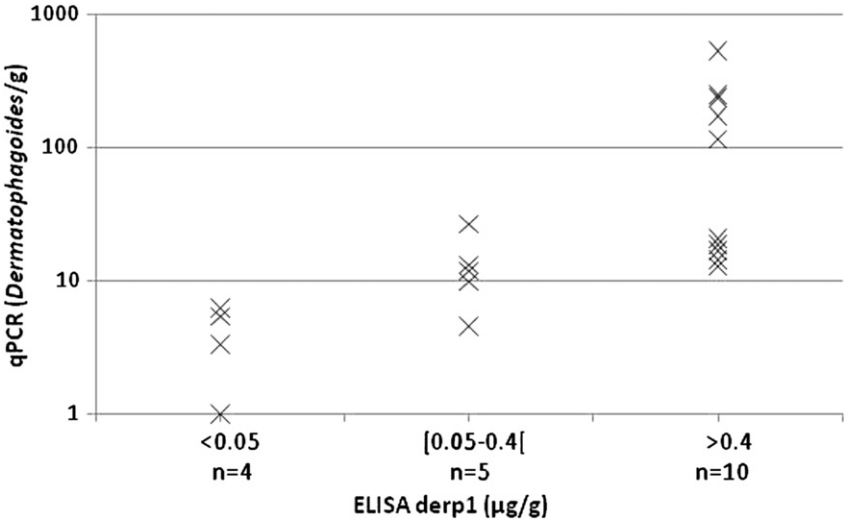


Fig. 2. Correlation between qPCR (DermaF1/R1/P1) and ELISA Der p 1 assays in mattress dust (n = 19).

We validated new qPCR assays to quantify house dust mites that have an important role in asthma and in the development of other allergic diseases. It would be useful to measure levels of exposure in epidemiological studies on human health.

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