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Assessment of pets (cats and dogs) in homes using electrostatic dust collectors and QPCR: new tools to evaluate exposure and risk of allergies

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ABSTRACT

Contradictory results are found in the literature concerning fungi, bacteria, and pet exposure and the risk of developing asthma. All these allergens have been thoroughly studied separately in cohort studies, and a variety of sampling and analytical methods are used. It is already possible to characterize fungi, mites, and bacteria by QPCR. The aim of our study is to evaluate QPCR systems to quantify the presence of cats and dogs in homes. Twenty-four houses were sampled with an Electrostatic Dust Collector which was analyzed by QPCR. Questionnaires on the presence of pets in homes were completed. The results from QPCR were correlated for real presence of cats and dogs, and highlighted indirect exposure. This study provides a useful screening tool that will be used in future large cohort studies, such as the ELFE cohort study.

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KEYWORDS

QPCR; housing; cat; dog; antigens

Introduction

Asthma has become the most common chronic childhood disease in the industrialized world (Eder et al. 2006). Asthma is described as an environmental disease resulting from the interaction between genetic predisposition and environmental exposure to pets, house dust mites (HDM) and molds, particularly indoor pollution, during the early years of life.

Many studies have attempted to explain environmental involvement in the development of allergic diseases. Real-time quantitative PCR (QPCR) technologies have highlighted a number of fungi involved in allergy or sensitization (Vesper et al. 2008), but their precise involvement remains inconclusive. Although some teams have identified certain fungi species that are correlated with a high risk of developing asthma (Reponen et al. 2012; Sharpe et al. 2015), the role of the different agents and mechanisms is unknown. Cohort studies have reached different conclusions which remain a challenge due to the multifactorial characteristics of asthma, and to the heterogeneity of the methods used for indoor characterization. Questionnaires may be used instead of sampling; samples may include active dust, passive dust or air. These samples may be quantified in culture or with PCR, or through antigen measurements. It is also difficult to time these measurements accurately, as samples may vary

considerably at different stages of a child's life, and may be taken once or repeatedly. Therefore, the available data cannot be compared and the conclusions of the different studies are often incomplete because they are so diverse.

QPCR is a useful technique for environmental micro-organism characterization and quantification and has recently been used in numerous cohort studies. The main advantages are accurate quantification, efficacy, sensitivity, and reproducibility. QPCR has been used by several teams (Kaarakainen et al. 2009; Kaarakainen et al. 2011; Yamamoto et al. 2011; Méheust et al. 2012; Reponen et al. 2012; Vesper et al. 2013) but in all these studies, QPCR was restricted to detection and quantification of molds and bacteria.

The ELFE study (Etude Longitudinale Française depuis l'Enfance), which started in 2011, consists of a 20,000 birth cohort study conducted on a multidisciplinary basis. Several working groups have been established, one of them focusing on asthma and allergic respiratory diseases. This particular group required a tailored system for taking measurements.

In the first step of this study, ten specific QPCR targets (six fungal species, one family and two genera of bacteria, one house dust mite) were used to analyze the micro-organism composition of an electrostatic dust fall collector (EDC) from 3193 dwellings in the ELFE cohort study (Rocchi et al. 2015).

This association of EDC over a period of several weeks and QPCR has previously been validated in a published pilot study. It best meets the needs of large cohort sampling. EDC is both discreet and cost-effective. It was well accepted by the participants (71% return in the pilot phase). When the sampling period was longer, biodiversity was increased and QPCR signal was slightly greater. The extraction methods and duplex QPCR were adapted for a minor cost (Scherer et al. 2014). Moreover, multivariate analyses from 3193 dwellings in the ELFE cohort study showed that the microbial composition of dwellings could be expressed in clusters, which seems promising for the evaluation of allergy risk (Rocchi et al. 2015). These profiles, which synthesize micro-organism diversity (regardless of the type of living organism) and reflect the complexity and interaction of a panel of micro-organisms in dwellings, could enhance our knowledge of the relationship between the microbiological environment and allergic diseases.

Because our results are thus far encouraging, it would be useful to improve these profiles by adding other allergenic agents which do not come from micro-organisms, i.e. pets, using the same DNA extracts and methods.

The relationship between exposure to dogs and cats in early childhood and the incidence of allergies is still controversial (Chen et al. 2010; Lodge et al. 2012). It has been suggested that contact with pets may increase the risk of allergic diseases and sensitization (Pyrhönen et al. 2015), or protect against them (Gaffin et al. 2012). Allergen measurements correlated with the presence of animals at home (Almqvist et al. 2003), and questionnaires are the current methods used to determine pet exposure in cohort studies (Crawford et al. 2015).

The purpose of our study was to assess QPCR techniques that were previously developed to detect cat and dog DNA in samples of feces containing parasitological diseases (Knapp et al. 2016). We aimed to measure exposure to pets in dwellings by obtaining quantitative results that can be integrated in the profile model previously used to measure environmental conditions in dwellings from the ELFE cohort.

The final step would be to establish a unique result in the form of a profile synthesizing the allergen mixture of each dwelling. This will include micro-organisms, mites, and pets, all of which could be related to health data of children from the ELFE Cohort.

Materials and methods

QPCR development

QPCR system for pets

The system was developed to identify carnivore feces using the high sensitivity of the real-time PCR technique. Probes and primers were previously designed for dogs and cats using the Primer Express

Table 1. Primers and hydrolysis probes designed to identify cats and dogs by QPCR.

Target	Primer & probe	Sequence (5'–3')	References
Cat (<i>Felis catus</i>)	Forward primer	CCCTTCTAGGAGTCTGCCTAATCTT	Knapp et al. 2016
	Reverse primer	CGGTTATTGTGCTGATGTGTAGTGT	
	Probe	(FAM)AAATCCTCACCGGCTCTTTTGCC(TAMRA)	
Dog (<i>Canis lupus familiaris</i>)	Forward primer	CCACCCACTAGCCAAAATTGTT	Knapp et al., 2016
	Reverse primer	AAGTTCCATCAAGCAGAGATGTTAGA	
	Probe	(VIC)ATAACTCATTGACCTCCCAGCGCC(TAMRA)	

3.0 for the host fecal test (Knapp et al. 2016), shown in Table 1. They are labeled FAM and VIC and are to be analyzed in duplex.

Efficacy, limit of detection and specificity

DNA from the epipdydim and ear tip was extracted using the High Pure PCR Template Preparation kit (Roche Diagnostic[®], Penzberg, Germany), as recommended by the manufacturer and was used to define the calibration scale of dogs and cats, respectively. The concentration of the first calibration point was 50 pg/μL.

For each of these targeted species, DNA extract was diluted from 50,000 to 0.5 fg/μL. Each calibrating point was run seven times. Limit quantification is defined as the smallest amount of DNA in which seven successful detections are observed in seven QPCR trials (Hospodsky et al. 2010).

Specificity was tested using DNA extract from 52 indoor micro-organisms currently found in houses and belonging to groups of mold ($n = 38$), actinomycetes ($n = 4$), yeasts ($n = 4$), mycobacteria ($n = 2$), enterobacteria ($n = 1$), and dust mites (HDM) ($n = 3$). DNA concentration in the sample tested was 500 fg/μL, except for dust mites (10 fg/μL). Cultivable micro-organisms were cultivated on adapted media at the correct temperature and extraction was performed as previously described (Scherer et al. 2014).

QPCR analysis

QPCR reaction was performed in a final volume of 20 μL, containing 10 μL of Hydrolysis Gene Expression Master mix 2X (Applied Biosystem, Foster City, CA), 1000 nM primers et 200 nM probe, and 5 μL of DNA extract. The amplification protocol was as follows: two minutes at 50 °C, 10 min at 95 °C for the activation, followed by 45 cycles for 15 s at 95 °C for template denaturation and one min at 60 °C for primer and probe annealing and extension. PCR reactions and absolute quantification were run and monitored using Applied Biosystems[®] 7500 Fast System (Life Technologies[®], Carlsbad, CA, USA). Sterile distilled water samples were used as negative controls in each series of amplification. The results are expressed with the Quantification Cycle, C_q . Negative results are expressed as $C_q = 45$.

Positive and negative controls

Four wipes from non-exposed EDC, two wipes rubbed on a dog nose and two wipes rubbed on a cat nose for five seconds were analyzed as samples.

Application of QPCR on dwellings with and without pet occupation

Sampling and QPCR Analysis of dwellings

Twenty-four houses were recruited in eastern France (Franche-Comté region). No environmental or medical criteria for selecting the type of dwellings were used. An Electrostatic Dust Collector (EDC) was exposed to the air on a horizontal surface between 1.60 and 1.80 m above the floor in an occupied room. Dust was allowed to settle over a six-week period in winter. A questionnaire on the type of housing and the presence of pets was given to the participants. Participants returned the questionnaires and the EDC at the end of the sampling period.

Table 2. Detection limits and repeatability of the two targets used in QPCR; correlation coefficient of the scale (r^2), mean of the Quantification Cycle obtained (C_q mean), Standard Deviation of these C_q ($SD C_q$), number of successful trials ($n = 7$) for each species.

	C_q mean	$SD C_q$	Number of success	C_q mean	$SD C_q$	Number of success
Conc (fg/ μ L)	Dog (<i>Canis lupus familiaris</i>) ($r^2 = 0.992$)			Cat (<i>Felis catus</i>) ($r^2 = 0.997$)		
50000	25.2	0.02	7	26.3	0.023	7
5000	28.7	0.085	7	29.6	0.049	7
500	32.2	0.072	7	33.0	0.206	7
50	35.5	0.30	7	36.5	0.391	7
5	39.0	0.98	7	39.5	0.466	5
0,5	39.8	1.93	2	nd	nd	0

Note. nd = absence of signal.

In bold: concentrations chosen for limit of quantification

EDC washing step, and DNA extraction from the EDC were performed as previously described (Scherer et al. 2014). All manipulations were carried out under ventilated hood with free DNA materials. Each extraction series included a blank extraction, and each amplification series a blank amplification.

DNA from cats and dogs was amplified using the QPCR assays described above.

Estimation of real exposure to pets in homes

Participants had to respond not only to questions about the presence of cats or dogs in their homes, but also about the number of pets and the frequency of their presence in the house and in the room tested (Direct Exposure). Using all of this information, three categories of exposure were created. The cat(s) or dog(s) was identified with the letter P if the animal was often in the tested room, and with the letter A if the pet was never in the tested room. In cases where animals were often in the house but rarely in the tested room, the letter M was used.

Participants also had to give information about regular contact with relatives who had a cat or dog at home (considered as Indirect Exposure (Almqvist et al. 2003)).

Statistical analysis

Non-parametric tests (Wilcoxon rank sum test and Kruskal–Wallis test) were used to investigate possible differences in C_q results, the presence or absence of pets at home and type of exposure, and the category of exposure. Statistical analyses were performed using R 2.13.1 (R Development Core Team, 2012).

Results

QPCR efficacy

Repeatability is expressed by the C_q standard deviation (Bustin et al. 2009). For each species targeted, mean C_q , C_q Standard Deviation ($SD C_q$), and the number of successful trials are reported (Table 2). The lowest concentration, with seven successful trials and $SD C_q < 1$, was considered as the limit of quantification. The quantification limit was about 5 fg DNA/ μ L for dogs, corresponding to $C_q = 39.0$. For cats, we considered $C_q = 39.5$ as the limit of detection because the sample with 5 fg/ μ L was detected in the 5/7 run, and the sample with 0.5 fg was not detected at all.

QPCR specificity

The results of QPCR analyses using the primers and probes designed for cat and dog detection were negative ($C_q > 40$ cycles) when applied to DNA extract from the 52 micro-organisms analyzed in specificity tests.

Table 3. Distribution of housing according to categories of exposure: number of each pet, completed by A = pet never in tested room, M = presence at home but rarely in tested room, P = frequent presence in tested room.

Exposition category	0	1A	1M	1P	2M	2P	3M	3P
Cat								
Number of dwellings	13	0	1	7	1	1	0	1
Dog								
Number of dwellings	14	3	1	5	0	0	1	0

Table 4. Number of animals, Exposure category and C_q results for each of the 24 samples.

Name of sample	Declared number of cats	Exposure category of cat	Mean C_q for cat QPCR	Declared number of dogs	Exposure category of dog	Mean C_q for dog QPCR
H1	0	0	45.0	0	0	45.0
H2	0	0	45.0	0	0	36.7*
H3	0	0	45.0	1	1P	35.5
H4	1	1P	36.3	1	1M	34.9
H5	1	1P	39.5	1	1A	39.4
H6	2	2M	35.1	3	3M	34.9
H7	0	0	45.0	0	0	40.6
H8	0	0	45.0	1	1P	29.4
H9	1	1M	38.8	0	0	40.6
H10	1	1P	36.9	0	0	45.0
H11	1	1P	36.6	0	0	38.1*
H12	1	1P	34.9	0	0	39.2
H13	0	0	45.0	0	0	40.6
H14	0	0	45.0	1	1P	33.7
H15	0	0	45.0	0	0	36.3*
H16	2	2P	32.8	0	0	39.7
H17	0	0	37.7*	0	0	37.5*
H18	3	3P	33.4	1	1P	32.3
H19	1	1P	37.2	1	1P	31.6
H20	1	1P	35.4	0	0	39.2*
H21	0	0	45.0	0	0	45.0
H22	0	0	45.0	0	0	36.9*
H23	0	0	45.0	1	1A	37.0
H24	0	0	45.0	1	1A	39.2

Note. Grey cases correspond to sample with positive signal without the pet concern contact.

*Identify the sample where indirect exposure has been reported.

QPCR positive and negative controls

No positive results were observed for the negative control of amplification and extraction.

No positive results were observed for the four wipes from non-exposed EDC.

The two 'dog wipes' were positive with $C_q = 32.92$ – 32.97 and negative for cat detection.

The two 'cat wipes' were positive with $C_q = 30.30$ – 32.86 and negative for dog detection.

Dwelling recruitment

Eighteen houses were in urban areas, and six in rural areas. The mean time of exposure was 45 days (minimum 24 days, maximum 60 days).

Of the participants, 46% declared having at least one cat, 42% at least one dog, 21% a cat and a dog. The number of dogs is divided into five categories, and the number of cats into six categories (Table 3).

QPCR on dwelling samples

Correlation between QPCR results and presence of pets in homes

The number of animals, exposure category, and C_q results are shown for each sample in Table 4.

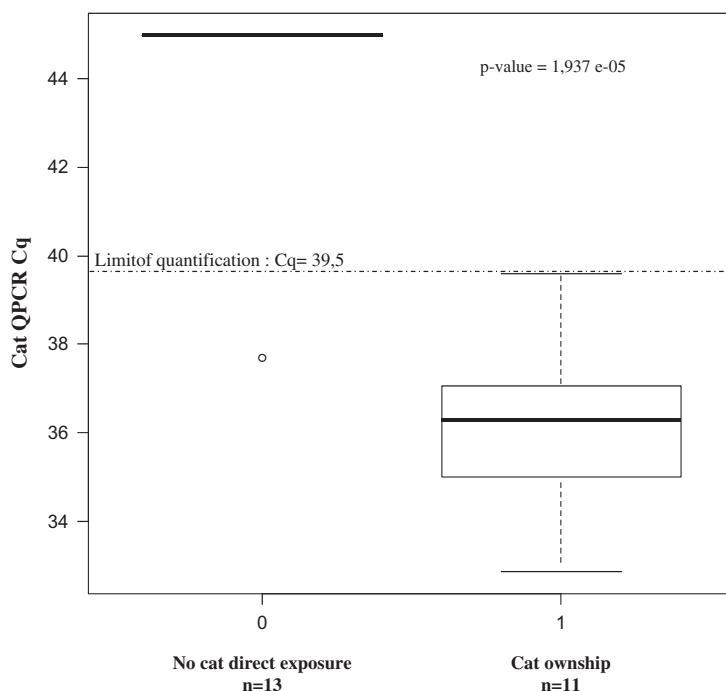


Figure 1. Boxplot with Cat QPCR results according to presence (1) or absence (0) of cat in the dwelling.

Cat QPCR was positive in 50% of houses, and dog QPCR was positive in 87.5% of houses. All houses with cats were cat QPCR-positive ($C_q \leq 39.5$ cycles).

A higher DNA concentration (i.e. lower C_q in QPCR) was measured in houses with the highest number of cats. Cat QPCR C_q was highly correlated with the presence or absence of cats (p -value = <0.0001) (Figure 1), and with the category of exposure (p -value < 0.005) (Figure 2). The QPCR signal was significantly higher (p -value < 0.01) when more than one cat was present (Figure 3).

Only one house without a cat presented a positive QPCR signal (H 17; $C_q = 37.7$), but indirect exposure was reported by frequent visits to and from families with cats and dogs.

All houses with dogs were dog-QPCR positive. The Dog- C_q is highly correlated with the presence or absence of dogs (p -value < 0.005) (Figure 4). Kruskal–Wallis was used to test the difference between categories of exposure in dog owners. The difference was statistically significant between Dog- C_q when dogs were present in the room (1P) compared to when they were never in the room (1A) (Figure 5).

In 11 cases, a positive QPCR signal was detected when no dogs were reported in the home. In six cases, the C_q was lower than 39 (our previously determined limit of quantification) but indirect exposure was reported. In the five other cases, C_q was higher than 39.2. The difference between dog QPCR signal when indirect exposure or no exposure was reported was statistically significant (p -value < 0.01) (Figure 6).

Discussion

When used together with EDC sampling, QPCR is a simple and accurate technique that can be applied to large numbers of samples. In this study, we confirmed the usefulness of QPCR techniques to quantify exposure to dogs and cats in houses. If we associate these new assays with fungi, bacteria and mite QPCR as previously described (Scherer et al. 2014), we will provide a new tool to quantify the overall allergenic risk in housing with the same sampling and analytical methods. The results will also be expressed in the same way.

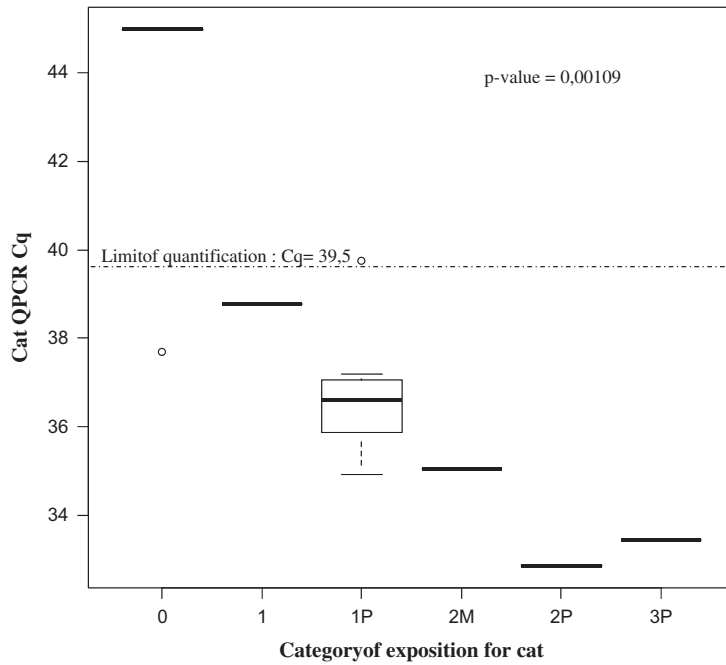


Figure 2. Boxplot with Cat QPCR results (in C_q – Inversely proportional to the concentration) according to the category of exposure (M = rarely in the room; P = often present in the room).

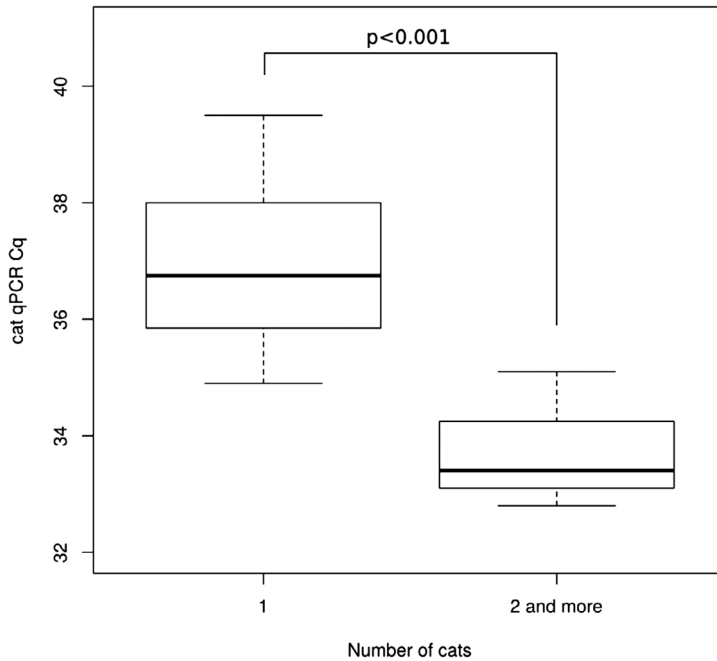


Figure 3. Boxplot with Cat QPCR results according to number of cats.

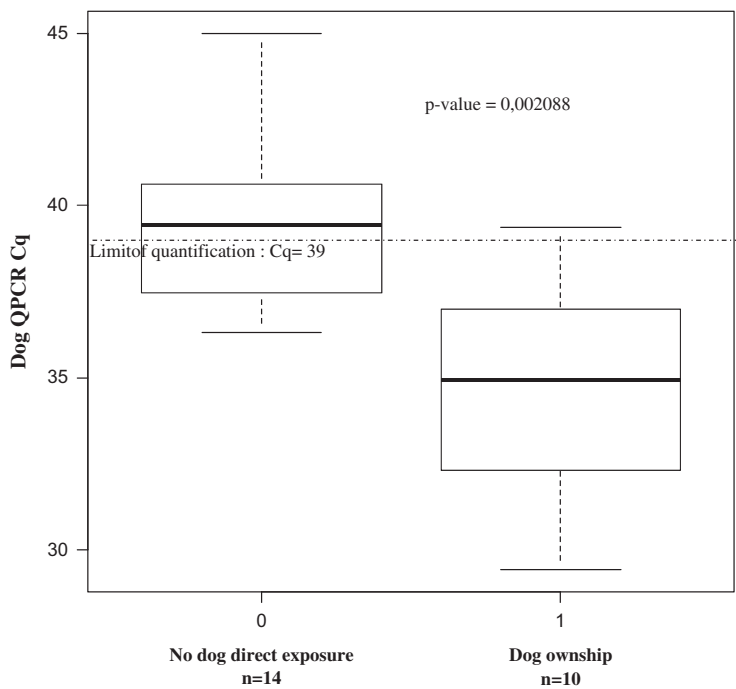


Figure 4. Boxplot with Dog QPCR results according to presence (1) or absence (0) of dogs in the dwelling.

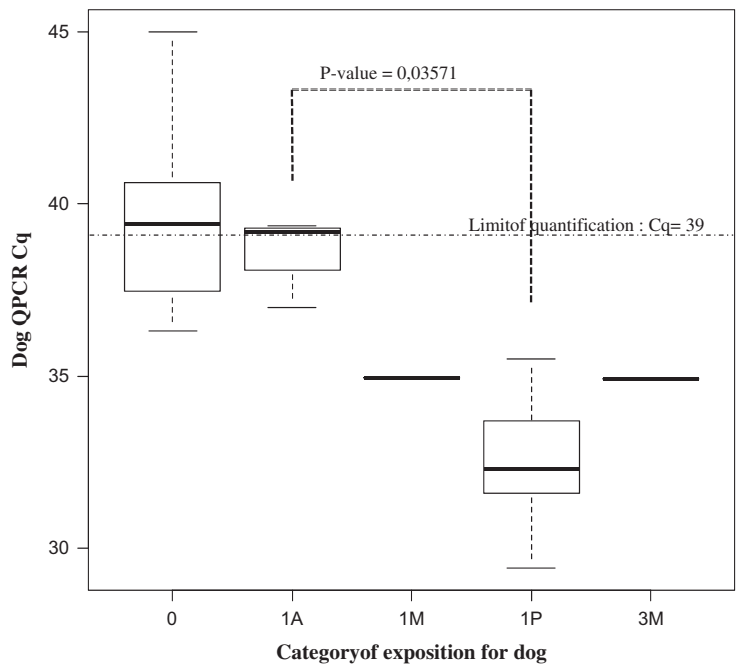


Figure 5. Boxplot with Dog QPCR results (in C_q – Inversely proportional to the concentration) according the category of exposure (A = never in the room; M = rarely in the room; P = often present in the room).

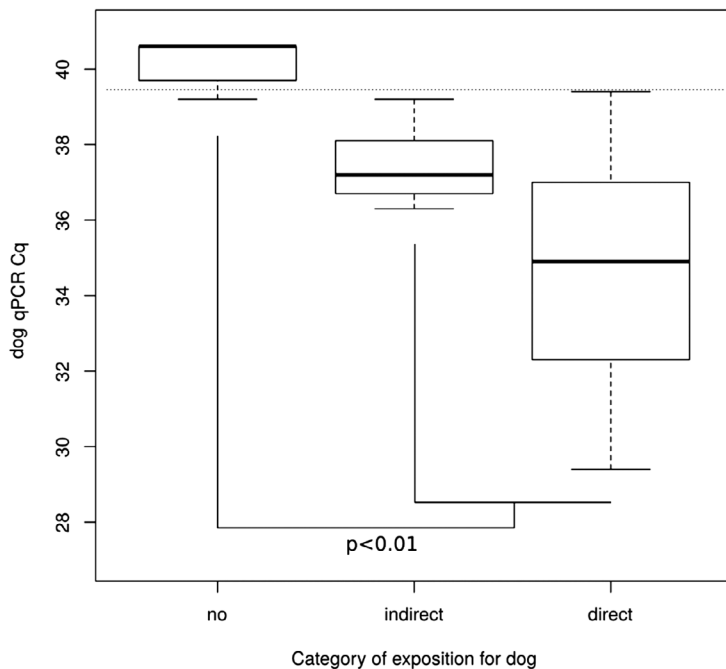


Figure 6. Boxplot with positive Dog QPCR results (in C_q – Inversely proportional to the concentration) according the type of exposure: no, indirect (outside contact but no dog residing permanently in the dwelling) direct (dog residing permanently in the dwelling).

The primers and probes system performed well, as shown by the analytical results. The quantification limit is set by seven out of seven successful detections, so it is strict in relation to the complex environmental situation. If we had applied this limit ($C_q = 36.5$; DNA concentration = 50 fg/ μ L) to cats, many of the results from houses would have been interpreted as unquantifiable, even though it corresponded to real exposure. The analytical limits of the method must be understood, but it is important to adapt these to the environmental situation. When results are between 36.5 and 39.5 cycles, in 5/7 cases, the real amount of cat DNA is successfully quantified. We therefore accept a limit of quantification $C_q = 39.5$ (DNA concentration = 5 fg/ μ L), knowing that the results from some houses may be false negatives.

The limit of quantification is 5 fg/ μ L ($C_q = 39$) for dog QPCR reaction, which seemed to correspond to the limit in terms of contact with dogs (direct and indirect) and a background signal, which could be an unquantifiable but detectable real signal.

The QPCR signal is correlated with the presence of animals at home, and with gradation linked to frequency of presence in the tested room and with the number of animals in the home. The correlation between the QPCR cat signal and category of exposure, the significant difference between having a dog indoors or outdoors and the ability to detect indirect dog exposure are convincing results. They show that this tool is effective for quantification and provides more detailed information than a questionnaire alone.

There seems to be a background dog QPCR signal. This background also exists when allergens are measured (Almqvist et al. 2003). In the Arbes study, 100% and 99.9% of houses tested positive for dog and cat allergens, respectively (Arbes et al. 2004). He suggests that the social environment is a major source of dog and cat allergens due to the allergen transport. Indeed, this is what we observe with a positive QPCR signal when indirect pet contact is reported. When the limit of quantification was higher, the signal usually highlighted a low presence of dog particles, lower than with known

indirect exposure. This should be interpreted as a positive result with no quantification. This is why it is essential to take measurements; questionnaires alone cannot accurately evaluate pet exposure.

We have developed a new tool which allows us to quantitatively measure pet exposure with a unique sampling and analytical technique. Associated with fungi, bacteria and mite QPCR as previously described, it provides a multi-parameter profile of the houses. This overall evaluation is needed to better understand the implications of chronic exposure, and to analyze the synergistic role of different micro-organisms and other allergens in the development of children's allergies. Another advantage of the proposed tool is that sampling can be done using EDC, which is very inexpensive and easy to use and does not require investigator intervention. Therefore, sampling can be repeated to better analyze early and later periods of childhood in longitudinal studies (Gaffin et al. 2014; Pyrhönen et al. 2015). Using the same techniques allowed us to express results with the same unit, and considerably reduce cost as well. Indeed, the sampling and extraction steps are the most costly and time-consuming. Adding two QPCR targets (one mix with the duplex) costs less than 1 euro per sample.

It is difficult to discuss the efficacy of extraction and the quantitative significance of the signal for pets. We cannot calibrate a 'pet' quantity for the wipe test in the same way as for fungi spore suspension, for example. That is why we chose to rub pets' noses. A Ct under 30 corresponds to a strong signal and large DNA quantity. Pets are not in direct contact with the EDC during the sampling period, so the C_t obtained with the EDC are coherent with our positive controls. These controls provided a scale to interpret our results.

Despite the small number of houses, these results are encouraging. The quantification of animals was well correlated with real exposure (unquantifiable by the questionnaire) and the analysis of these data, associated with other data on fungi, bacteria, and HDM could explain the interaction between the different organisms in the environment and help to understand the relationship between complex exposure and allergic diseases. The ELFE cohort study is collecting measurements from the dwellings of many children. The EDC has already been used once when the children were newborns and these children will be followed until they are aged 18. Another round of EDC sampling will be performed when the children are aged five. These new targets described in this article will be tested in the first frozen samples. Further investigations will be made to collect homogenous and quantitative data on allergenic exposure in dwellings.

We provide new QPCR assays to quantify pet exposure that have an important role in asthma and in the development of other allergic diseases. Integrated with QPCR fungi, bacteria, and mite QPCR already validated, it will be a useful tool to measure levels of exposure in epidemiological studies on human health.

Disclosure statement

No potential conflict of interest was reported by the authors.

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