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Review

Immunologic, spectrophotometric and nucleic acid based methods for the detection and quantification of airborne pollen

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ABSTRACT

Microscopic identification of pollen morphological phenotypes has been the traditional method used to identify and quantify pollen collected by air monitoring stations worldwide. Although this method has enabled a semi-standardized approach for the assessment of pollen exposure, limitations including labor intensiveness, required expertise, examiner bias, and the inability to differentiate species, genera, and in some cases families have limited data derived from the these stations. Recent advances in chemical, biochemical and molecular detection methods have provided standardized alternatives to this microscopic approach. In this review, we examine the applicability of alternative methodologies, in particular nucleic acid based assays involving the quantitative polymerase chain reaction, for the standardized detection of airborne pollen.

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Abbreviations: AAAAI, American Academy of Allergy, Asthma and Immunology;B/R ratio, blue:red autofluorescence;Ct, threshold cycle;FT-IR, Fourier transform infrared;HIA, Halogen Immunoassay;ITS, internal transcribed spacer;LOQ, limit of quantification;mAbs, monoclonal antibodies;NAB, National Allergy Bureau;rRNA, ribosomal RNA;PBM, protein binding membrane;PCR, polymerase chain reaction;qPCR, quantitative polymerase chain reaction.

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1. Introduction

Bioaerosols are defined as airborne particles with a biological origin (Eduard and Halstensen, 2009). Botanical sources include pollen, spores, trichomes, achene fibers, bark, seeds, and plant fragments. Airborne pollens are, in many cases, the most abundant plant-derived bioaerosols identified in samples collected from the outdoor environment. Unlike fungal conidia and hyphae which are viable propagules capable of saprobic colonization, pollens are not capable of growth outside compatible female gametophytes (Shukla et al., 1998). Lower plants including pteridophytes (ferns), bryophytes (hornworts, moss, liverworts), and algae may also produce pollen-like structures termed spores that can be aerosolized into the environment but often in much lower concentrations than pollen (Mandrioli et al., 2003).

The aerosolization of pollen occurs by two distinct dispersal mechanisms. Most gymnosperms and some angiosperms present cones and anthers to the wind (anemophilous). Abiotic disturbances (i.e. wind gusts) facilitate the dispersion of large concentrations of pollen into the prevailing wind current. Pollen may also be presented on anthers within a flower and dispersed by insects or other biotic pollination vectors (entomophilous). Abiotic disturbances to plants that utilize this pollination vector may also result in the airborne dispersal of pollen. Once aerosolized, the majority of pollen (~81% of *Pinus* pollen) deposits within 700 m of the source (Persson, 1954); however, some pollen may also be carried at long distances (Johansen and Hafsten, 1988; Rogers and Levetin, 1998) or even re-aerosolized following deposition (Igarashi, 1987; Green et al., 2003).

Airborne pollen concentrations are characterized by seasonal periodicity that is dependent on the phenology of the species as well as a number of well-characterized environmental parameters that promote plant growth and pollen development. Environmental surveys conducted throughout the world have provided detailed insight into the distribution (daily and seasonal) and airborne concentration of the most abundant pollen types, particularly those identified as etiological agents of seasonal hay fever and asthma. In the United States, allergic sensitization to pollens represents a major public health burden. Results from the Third National Health and Nutrition Examination Survey demonstrated that the prevalence of allergic sensitization was highest to dust mite (27.2%) and to seasonal pollen allergen sources including perennial rye (26.2%) and short ragweed (26.1%) (Arbes et al., 2005). To monitor the seasonal distribution of airborne pollen in the United States, the American Academy of Allergy, Asthma and Immunology (AAAAI) Aeroallergen Network established the National Allergy Bureau (NAB) to report to the public the concentration of pollen and mold spore levels from monitoring stations located throughout the country (Kosisky et al., 2011). However, this is just one example of a pollen monitoring network. There are many networks located throughout the world that are operated by individual researchers, aerobiologists that participate in collaborative networks, or by private industry. Typically, pollen is collected at each monitoring station using impaction samplers (Burkard volumetric spore trap, Kramer-Collins sampler, or Rotorod samplers) that are located on an unobstructed rooftop or 13 ft platform (refer to http://www.aaaai.org/global/nab-pollen-counts.aspx).

Impacted particles are often (but not always) stained to facilitate bright-field microscopic identification of pollen grains. Morphologically, pollens vary in size, shape, surface structures, and internal detail (Weber, 1998). The pollen wall consists of several layers; an inner wall composed of cellulose (intine) and an outer wall (exine) composed of sporopollenin, wax, and proteins. Apertures that allow passage of the pollen tube during pollination as well as surface wall architecture also characterize the outer wall of pollen. These phenotypes are used by the certified examiner to differentiate pollen to a given taxonomic level (family, genus etc.). Following classification, specific pollen types are quantified and calculated as pollen grains per m^3 of air.

Although optical identification/quantification of pollen bioaerosols has become increasingly standardized over the last few decades, there are a number of caveats associated with the current method of assessment. The microscopic identification of airborne pollen is labor and time intensive, requires a trained palynologist, and is subject to examiner bias. It is also challenging to differentiate pollen derived from prevalent aeroallergen species within a family; for example Poaceae (grass) pollen. In addition, fragments of plant material are also important from a sensitization/allergy perspective since they harbor allergenic epitopes, but are morphologically unidentifiable by microscopic examination (Taylor et al., 2007). These limitations highlight the benefits that might be realized using more objective and automated procedures for identifying and quantifying pollen. However, automated methods often require an initial selection of species to be quantified, which means that any species not chosen for quantification may be overlooked. In this review, alternative methods, with a particular focus on quantitative polymerase chain reaction (qPCR), will be evaluated.

2. Alternative methods

2.1. Automated morphological pattern recognition

Pollen is currently differentiated by palynological examiners who identify phenotypes using bright field microscopy

(Weber, 1998). During the last 20 years, palynologists and computer scientists have collaborated in an attempt to mechanize morphological identification through automated pollen recognition of microscopy images. Morphological characteristics that are considered in the identification process have included texture (Langford et al., 1990; Li et al., 2004), two and three-dimensional shapes (Boucher et al., 2002; Ronneberger et al., 2002) or a combination of the above (Zhang et al., 2004; Holt et al., 2011). Acquired images are automatically compared to a pollen image library to identify the pollen grain. The accuracy of this procedure when differentiating a small number of taxa has typically been high (90–97%); however, the challenge that remains is the translation of these experimental systems into instruments that can be widely distributed to pollen monitoring stations. Recently, Holt et al. (2011) described a fully automated pollen counter that provided reproducible results, and yet was cheaper than a conventional optical microscope, camera, and image analysis software used in traditional pollen assessment. To date, the capability of this instrument has only been tested on six species of varying pollen morphology. More rigorous testing will be required to determine whether this instrument can reproducibly identify and quantify pollen from heterogeneous aerosol samples, which are likely to contain a wide array of pollen species. Also, this automatic recognition system may fail to identify dehiscent and deformed pollen grains that are common in the atmosphere.

2.2. Spectroscopy

2.2.1. Raman spectroscopy

Raman spectroscopy employs a small beam of photons to gain chemical information from a given sample. Photons interact with molecular vibrations of a sample and yield scattered photons with different energy states. The emitted photon energies correspond to the molecular energies of the biomolecules in the sample (i.e. lipids, proteins, nucleic acids etc.). The resulting spectra can be used to assess the molecular vibrations of pollen grains, which is likely to differ among species. Previous studies have used this approach to confirm that near infrared (IR) Raman spectroscopy can distinguish various pollen species (Laucks et al., 2000; Schulte et al., 2008). Since typical pollen samples are likely to contain a heterogeneous mixture of particles, Schulte et al. (2008) employed near IR Raman microspectroscopy, which allows the user to apply a photon beam directly to a single pollen grain within the microscopic field of view. Spectra were recorded for over 90 different 'unknown' pollen grains that represented 15 tree species. Hierarchical cluster analysis was used to compare the 'unknown' spectra to a library of spectra generated with standard pollen stocks. In this study, 96% of spectra from "unknown" samples were correctly identified to species, except among species within the oak genus, which displayed homogenous spectra. While these results are promising, outdoor aerosol samples may exhibit more species heterogeneity than the ones tested in these studies and the pollen distribution would also be dependent on the geographic locale and time of year. Also, many allergenic pollen taxa (e.g. Poaceae) may exhibit homogenous spectra that would prevent genus or species level identifications.

2.2.2. Fourier transform infrared (FT-IR) spectroscopy

Similar to Raman spectroscopy, FT-IR spectroscopy is a method that uses the interactions between photons and the molecular vibrations of a sample to gain insight into the chemical composition of samples. Compared to Raman spectroscopy, FT-IR allows for wider spectra (4000–850 cm^{-1}) to be obtained than Raman (1600–400 cm^{-1}), allowing for more robust spectral analyses. FT-IR provides a reproducible and accurate method to differentiate pollens belonging to four morphologically indistinguishable species; however, each investigational sample was prepared as a pure homogenous sample (Pappas et al., 2003). To identify species within a heterogeneous pollen mixture, a microspectroscopy approach (similar to the Raman spectroscopy studies) was developed by several research groups (Gottardini et al., 2007; Dell'Anna et al., 2009). Using this approach, grain-to-grain variability in certain plant species was found to limit the applicability of this methodology. In addition to the infrastructure costs associated with an FT-IR, the acquisition of FT-IR spectra from tens (if not hundreds) of single pollen grains is labor intensive and would likely discourage its use in pollen monitoring stations (Dell'Anna et al., 2009). While Raman and FT-IR spectroscopy can eliminate some of the subjective biases associated with palynological identification, a significant time component remains, in addition to the requirement of sensitive and expensive instrumentation.

2.2.3. Autofluorescence

Pollen grains exhibit autofluorescence which is a property whereby pollen struck with an excitation wavelength emits light of a different wavelength (i.e. emission wavelength). Although an early study was not able to differentiate the autofluorescent signals derived from various pollen types (Laucks et al., 2000), recently Mitsumoto et al. (2009) described the differentiation of 9 pollen species based on the ratio of blue: red autofluorescence (B/R ratio). The authors also described the utility of the light scattering properties to distinguish the size of pollen grains (Mitsumoto et al., 2010). Additionally, Mitsumoto et al. (2010) tested the capability of real time pollen-identification using a flow cytometry platform. Importantly, the authors employed an aerosol sampler coupled with a particle separator that diverted objects of 1 μm or less away from the sampling chamber. This design allows for the diversion of only pollen-sized particles to the fluorometric chamber, thereby reducing the noise associated with non-target airborne particles. Proof-of-concept experiments resulted in the quantification of two pollen species (*Cryptomeria japonica* and *Chamaecyparis obstusa*) that were often misclassified when co-detected. Although autofluorescence coupled with flow cytometry offers some potential for pollen identification, using species-specific monoclonal antibodies (mAbs) as a labeled fluorescence source may supplement the capabilities of a real-time flow cytometry assay.

2.3. Immunoassays

The identification and characterization of prominent pollen allergens have enabled the production of high affinity mAbs. As a result, a variety of immunoassays that utilize different assay formats have been developed and enabled the quantification of pollen allergens in air samples (Holmquist and Vesterberg,

1999; Marth et al., 2004; Fernandez-Gonzalez et al., 2010). Clinically, the quantification of allergen may be more relevant than direct pollen counts especially as pollen fragments may be quantified (Taylor et al., 2007). However, the specificity of this approach is confounded by cross-reactive epitopes within or between botanical families. More than one major allergen can be associated with a species, and the availability of mAbs is often restricted to a prominent species. Recent developments in multiplex platforms have enabled the simultaneous detection of several indicator allergens for a given allergen species in one assay (Earle et al., 2007; Samadi et al., 2010). However, the success of this approach in a monitoring network format would be dependent on the availability of additional mAbs that are specific to other less characterized but regionally prevalent botanical genera.

An alternate immunodiagnostic platform that could be utilized to quantify pollen bioaerosols is the Halogen Immunoassay (HIA) (Tovey et al., 2008). In this indirect immunoassay, airborne pollen is collected onto a protein binding membrane (PBM) such as polyvinylidene fluoride or mixed cellulose ester. Overlaying the PBM with an adhesive backed tape or optically clear adhesive retains the airborne pollen on the surface of the PBM. The membrane can be immunostained with human IgE, mAb, or polyclonal antibodies. The immunostaining is observed microscopically as a halo around the particle. This approach was originally developed for the detection of perennial allergen sources but was later modified for the detection of airborne pollen (Razmovski et al., 2000). Like traditional methods, the HIA is confounded by examiner bias associated with the microscopic identification of pollen and it is a technically challenging immunoassay. Although this approach has been utilized in clinical studies and has provided unique insights into personal exposure to a variety of bioaerosol sources (Tovey et al., 2008), the HIA would not be a practical immunoassay platform for the rapid detection of pollen collected at a monitoring site.

2.4. Quantitative polymerase chain reaction (qPCR)

Variation in DNA sequences among different botanically sourced bioaerosols can be utilized for molecular species identification. One common method used to detect sequence variations is polymerase chain reaction (PCR). PCR is an in vitro enzymatic reaction where low quantities of a specific DNA sequence (i.e. “target DNA”) are amplified into larger quantities of the same sequence (Saiki et al., 1988). With standard PCR, the reactions are incubated on a programmable thermocycler and the final products are separated via agarose gel electrophoresis and stained with a fluorescent DNA binding agent such as ethidium bromide or SYBR Green. However, standard PCR cannot provide quantitative information about how much target DNA from a particular pollen source was present at the beginning of the reaction. In contrast, quantitative PCR (qPCR) measures the extent or magnitude of the amplification of target DNA in real-time through the use of fluorescent DNA-binding dyes (e.g. SYBR green) or sequence-specific fluorescent probes and a thermocycler equipped with fluorophore reading capabilities (Gibson et al., 1996). In general, sequence-specific fluorescent probes are preferred over DNA-binding dyes in qPCR because they add another layer of specificity to DNA quantification, and they also are less likely to generate false positive results. Coupled with the establishment of a standard curve, inferences can then be made about the starting concentration of target DNA based on the cycle at which the baseline fluorescent threshold is exceeded (i.e. the cycle threshold, or Ct, value).

As qPCR instrumentation and reagents become more available, this standardized molecular procedure has supplemented (with the potential to replace) traditional methods of bioaerosol assessment. qPCR has been used to quantify a variety of airborne bioaerosols, including viruses (Blachere et al., 2007), bacteria (Dutil et al., 2007), fungi (McDevitt et al., 2004), and pollen (Longhi et al., 2009). Considering the expertise required to correctly identify/quantify pollen, qPCR can potentially provide a more objective and standardized approach that may facilitate automated analysis and the expansion of pollen sampling sites (Longhi et al., 2009). The following sections provide a brief overview of the necessary considerations for pollen quantification using qPCR, including preliminary investigations, a potential pollen analysis protocol, infrastructure requirements, cost analyses, and potential limitations.

3. Potential of qPCR for pollen monitoring

3.1. Preliminary experiments

Fig. 1 illustrates the preliminary steps required before qPCR could be employed for the quantification of pollen bioaerosols. In the first step, 15–20 plant genera and/or species (predominantly anemophilous tree, weed, and grass species that broadly represent etiological agents of pollen which induce allergic rhinitis in the U.S.) would need to be selected for quantification. These species would not require uniform distribution (i.e. different species that are present in Colorado versus Florida); however, for economic reasons, each sampling site should be limited to 15–20 different species (i.e. ‘targets’). When appropriate, “genus-specific” primer/probes sets should be attempted. For example, one primer/probe set that recognizes all allergenic *Betula* species may be desirable compared to developing separate primer/probe sets for each individual *Betula* species. Determination of the appropriate genera/species could be based on historic phenological and aerobiological data from each sampling station. Following species selection, a DNA extraction protocol will need to be optimized for extraction efficiency, removal of PCR inhibitors, and detection of pollen that may be present in low concentrations. Commercial DNA extraction kits are available and several are specific for plant-derived material. These kits contain various cell lysis methods that would need to be tested empirically on standard pollen stocks and the resultant DNA quantity measured with highly sensitive fluorometric techniques (e.g. PicoGreen kit, Invitrogen, CA). Different methods of pollen collection (i.e. Burkard-tape vs. Rotorod vs. cyclone type samplers) would also need to be assessed for their efficacy in collecting pollen samples suitable for DNA extraction (i.e. potential interference of the chemicals in the adhesive tape). Since the Burkard-tape and Rotorod samplers are common worldwide, it may be worthwhile to empirically test whether or not they can be adapted for molecular analyses such as qPCR.

In order to develop a reproducible qPCR quantification assay for pollen, a genomic DNA region must be selected as an amplification target. While several genomic targets are

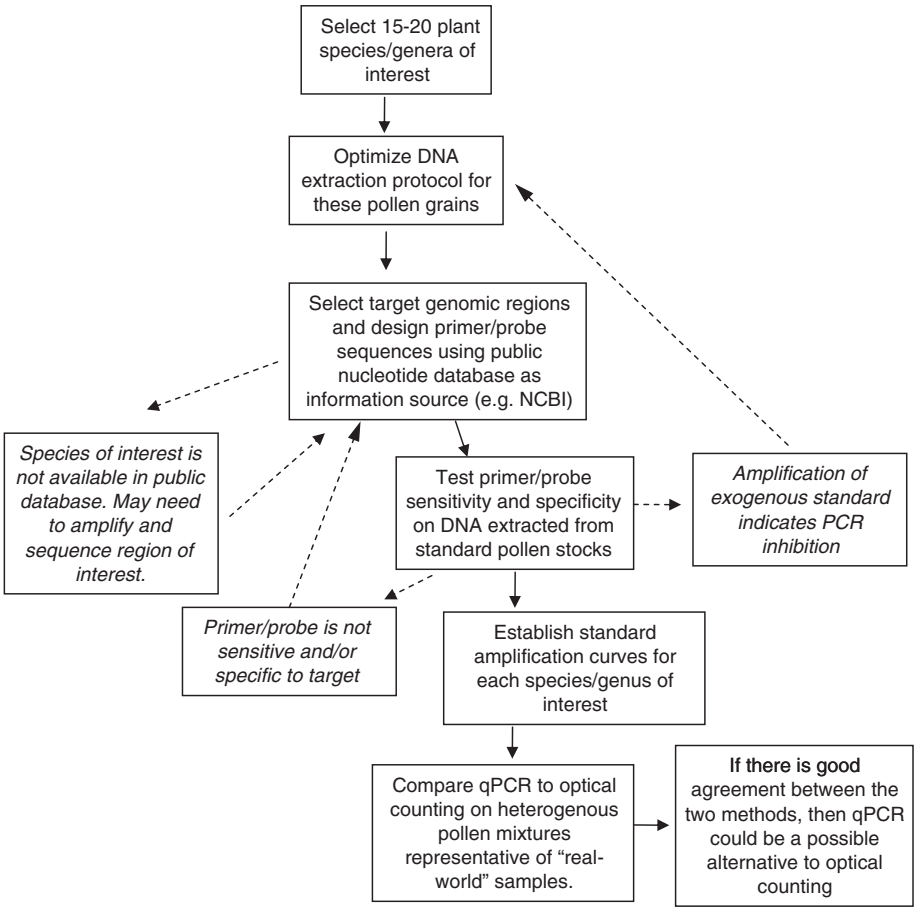

Fig. 1. Flowchart describing the steps that must be taken during preliminary experimentation. Dotted arrows and italicized text represent possible limitations that may arise. Limitations may be overcome by revisiting the previous step and optimizing the conditions.

suitable (see Longhi et al., 2009), the internal transcribed spacer (ITS) regions of nuclear ribosomal RNA (rRNA) genes are often preferred since ITS sequences for many species are available in public databases (e.g. http://www.ncbi.nlm.nih.gov/nuccore). Another advantage of targeting ITS regions is that several copies of the nuclear rRNA gene are typically present within the genome. This improves the likelihood of amplification of samples containing small quantities of pollen. However, since ITS regions are not part of the final rRNA molecule, they also mutate very quickly. ITS sequence (but not in the rRNA) hypervariability may result among closely related species and this highlights the importance of testing the specificity of the designed primer/probes.

Following the selection of a target DNA region, qPCR primer/probe sets would need to be designed for all genera/species of interest. Each set will contain two primers (forward and reverse) and one probe. Depending on the availability of target sequences in public nucleotide databases, designing primer/probes may be as simple as analyzing the sequences with a software package such as Primer Express 3.0 (Applied Biosystems). Software packages have the capability of selecting the optimum primer/probe oligonucleotides based on their thermodynamic stability. These primer/probe sequences can then be tested in silico for their specificity to a given genus/species by searching for homologous sequences in public databases. One possible limitation that might be encountered using this approach is the lack of sequence availability for a species of interest. In this case, universal plant primers can be designed and the ITS region of the species of interest can be amplified and then sequenced. The acquired sequence data can then be queried for an optimum primer/probe sequence for that particular species.

Once all primer/probe sets are designed, they will need to be tested for their specificity against all species of interest. Template pollen obtained commercially would be preferable. Pollen that is not commercially available may have to be obtained and purified from field samples. Pollen collection could be conducted during the flowering interval of those particular species of interest. If the selected primer/probes cross-react between unrelated species, new primer/probe sets would need to be designed and tested for specificity using the same stock pollen sources.

Following the development of primer/probe sets and characterization of specificity, DNA would be collected from serial dilutions of pollen, each processed using the qPCR with respective primer/probe sets to develop a set of standard curves. At this point, the limit of quantification (LOQ) may be determined (e.g. 100 pollen grains) and will provide critical information about the utility of the qPCR for quantifying airborne pollen. Once standard curves have been established for each primer/probe set, a proof-of-concept experiment would need to be

performed to compare the current microscopic quantification methods to qPCR of spiked pollen samples (e.g. pollen grains of different species applied to sampling adhesive). If these results correlate and are reproducible, then qPCR may be a technically (though not necessarily economically) alternative approach to microscopic quantification of pollen.

3.2. Analysis protocol

After conducting preliminary experiments and establishing standard curves, the quantification of pollen from air samples will require four steps (Fig. 2). The first step includes the collection of pollen bioaerosols for DNA extraction. To date, other researchers have successfully extracted DNA derived from pollen collected on aerobiological tape samples (Longhi et al., 2009). The optimum DNA extraction method (as determined in preliminary experiments) would then be used to isolate DNA from collected pollen. The DNA would then be added to premixed qPCR reactions previously aliquoted into 96-well PCR plates containing primer/probe sets for the various species of interest. After the qPCR program has run, the Ct values would be inserted into the predetermined standard curve equations to calculate the pollen loads of each sample. The total amount of time required to extract and run the qPCR is ~2.5 h (the duration of individual steps is included in Fig. 2).

3.3. Possible limitations

3.3.1. PCR inhibitors

Several organic substances can inhibit qPCR enzymatic reactions and these are reviewed by Wilson (1997). These PCR inhibitors may accumulate during air sampling and potentially inhibit the qPCR and cause skewed values. In addition, the pollen grains themselves have also been shown to inhibit PCR (St. Pierre et al., 1994). Inclusion of an exogenous standard, such as the yeast species, *Geotrichum candidum*, would help monitor and assess PCR inhibition (Haugland et al., 1999). To overcome several of these limitations, pollen DNA extraction methods should be tested for their capabilities to remove PCR inhibitors in preliminary experiments.

3.3.2. Limit of quantification (LOQ)

In a previous study, the lowest quantity of pollen grains detected using qPCR was ~100, approximately ten fold higher than the minimum number that could be detected using microscopic counting methods (Longhi et al., 2009). When testing qPCR for pollen analysis, the authors targeted single-copy genes due to improved conservation within species (Longhi et al., 2009). Using multi-copy ITS regions as targets may increase the sensitivity of the qPCR assays to levels that are comparable to microscopic methods. The LOQ may also be influenced by variables such as the collection method, as DNA may be more efficiently extracted from one sample type (e.g. aerosol tube) versus another (e.g. tape). The effects of the sampling method and the LOQ for each species of interest will need to be determined during preliminary experimentation. Very low pollen concentrations (<10 grains per sample) may not be detected using qPCR due to the inherent limitations of extracting low quantities of DNA. However, preliminary data will indicate whether or not qPCR can reach LOQs comparable to microscopic pollen monitoring methods.

3.3.3. Inherent DNA variability within species

Although targeting the ITS region of rRNA genes has several advantages (sequence availability, high-copy number within genomes), one disadvantage is the higher-than-normal mutation rate in ITS sequences. The high mutation rate may cause variability in ITS sequences among pollen derived from the same species, which would hinder primer and/or probe hybridization. This would limit the detection of pollen grains where the ITS sequence has mutated and cause false-negative qPCR results. Using ITS sequences from as many representatives of a target species will help identify conserved regions during the design stages of primers and probes. In addition, testing primer/probe sets on as many pollen samples (commercial or collected) as possible during preliminary investigations will help determine whether ITS sequence variability will confound the analysis. Although slight variations in ITS sequence within species are common, this has not been identified as a limitation in qPCR approaches for fungi (Vesper et al., 2007) or bacteria (Dutil et al., 2007).

3.4. Feasibility

Based on the current prices of qPCR reagents, quantifying pollen from 18 different plant species would approximately cost $9000 USD per year per sampling station. This estimate does not include the cost of preliminary experiments, labor, or infrastructure (sampling equipment, qPCR thermocycler etc.). There are currently ~85 sampling stations reporting to the NAB monitoring network, all of which are self-funded (refer to http://www.aaaai.org/global/nab-pollen-counts.aspx). Each station may find the cost and infrastructure required to perform these analyses cost-prohibitive. One possibility at alleviating costs would be establishing 5–10 "qPCR regional centers" that receive the samples from multiple stations, extract DNA, and perform the qPCR analyses. However, the caveat with this approach would require a significant extramural funding source for the establishment and management of external qPCR centers. This option would also introduce a lag time in the reporting of pollen counts that may preclude their clinical use in providing real-time information about the types and quantities of airborne pollen to allergic patients.

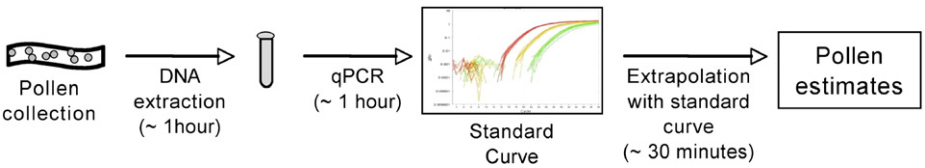

Fig. 2. Overview of qPCR quantification of pollen. The figure indicates the estimated time required for each step.

4. Conclusions

Although several alternative techniques described in this article have the potential to replace microscope pollen quantification methods, they have associated limitations (both technical and economical) that may initially preclude their adoption in pollen monitoring networks. The self-funding nature of pollen stations (at least in the U.S.), coupled with the widespread standardization of microscopic pollen quantification, provides little incentive for a shift from current microscope based protocols. As these alternative technologies evolve and become economically viable options in the future, these modern methods have the potential to be adopted by international monitoring networks and provide a standardized approach to quantifying airborne pollen.

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