Detection and Quantification of *Wallemia sebi* in Aerosols by Real-Time PCR, Conventional PCR, and Cultivation

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Received 24 March 2004/Accepted 16 July 2004

Wallemia sebi is a deuteromycete fungus commonly found in agricultural environments in many parts of the world and is suspected to be a causative agent of farmer's lung disease. The fungus grows slowly on commonly used culture media and is often obscured by the fast-growing fungi. Thus, its occurrence in different environments has often been underestimated. In this study, we developed two sets of PCR primers specific to *W. sebi* that can be applied in either conventional PCR or real-time PCR for rapid detection and quantification of the fungus in environmental samples. Both PCR systems proved to be highly specific and sensitive for *W. sebi* detection even in a high background of other fungal DNAs. These methods were employed to investigate the presence of *W. sebi* in the aerosols of a farm. The results revealed a high concentration of *W. sebi* spores, 10^7 m⁻³ by real-time PCR and 10^6 m⁻³ by cultivation, which indicates the prevalence of *W. sebi* in farms handling hay and grain and in cow barns. The methods developed in this study could serve as rapid, specific, and sensitive means of detecting *W. sebi* in aerosol and surface samples and could thus facilitate investigations of its distribution, ecology, clinical diagnosis, and exposure risk assessment.

Wallemia sebi is a deuteromycete fungus capable of growth over a wide range of water activity from 0.69 to 0.997 (15). It can potentially grow in various environments and on different substances and has been isolated from jam, cake, cereals, salted meat, fish, and dairy products (12, 23). Up to now, only one species is described in the genus *Wallemia*. *W. sebi* grows slowly on commonly used culture media, such as malt extract agar, and is often obscured by the fast-growing fungi. Thus, its presence in different environments has often been overlooked, which in turn hindered the studies on its distribution and ecology. Recently, with the use of selective media for xerophilic fungi, *W. sebi* has been found to be very common in the agricultural environments of many parts of the world (4, 6, 9, 16).

The conidium of *W. sebi* has a shape of a rough-surfaced sphere of 2.5 to 3.5 μ m in diameter (18); thus, it can reach the respiratory bronchioles when inhaled. Airborne *W. sebi* has been suspected to be a causative agent of human allergies, particularly bronchial asthma (17). Elevated levels of immunoglobulin G (IgG) antibodies were observed among Finnish farmers exposed to *W. sebi* (9). In eastern France, *W. sebi* has also been identified as playing a role in farmer's lung disease (16). The fungus produces a toxic metabolite, walleminol A, with a bioinhibitory dose effect similar to those of other mycotoxins such as penicillic acid (23).

Conventional methods for the detection and quantification of *W. sebi* rely on microscopic or culture techniques that are time consuming and laborious. Molecular techniques are promising approaches complementary to the conventional detection methods. PCR-based methods have the advantage of

detecting the presence of microorganisms in a sample regardless of their culturability at the time of analysis. Recently, the introduction of real-time PCR by including a fluorescent dye reporter in the reaction has offered the ability of simultaneous detection and quantification of DNA of a specific microbe in one reaction. This technique is faster than the conventional PCR by excluding post-PCR gel electrophoresis and has become popular in ecological and environmental microbiology and clinical diagnosis (2, 11, 13).

In this study, we aimed for the development of a rapid and sensitive method for the detection and quantification of *W. sebi* in aerosol samples from agricultural environments. Based on 18S rRNA gene sequence data, specific PCR primers were designed to selectively amplify *W. sebi* from composite environmental samples. These primers can be used in both conventional PCR and real-time PCR detections. The detection specificities and sensitivities of the two PCR systems were compared. The validated real-time PCR system was applied to the detection of *W. sebi* in aerosols from a farm in northern Sweden. The concentration of *W. sebi* derived from the real-time PCR was compared to culture-based CFU counting. The analytical methods developed in this study could facilitate the rapid detection and quantification of *W. sebi* in environmental samples, thus providing information about its distribution and ecology.

MATERIALS AND METHODS

Fungal strains and genomic DNA extraction. One strain of *W. sebi* (UPSC 2502) was obtained from the Uppsala University Culture Collection of Fungi (Uppsala, Sweden) (Table 1). Another 30 strains of *W. sebi* were isolated from outdoor air in the suburbs of Beijing, China, and northern Sweden. These strains were identified through cultivation on dichloran–18% glycerol (DG18) agar (Oxoid, Basingstoke, United Kingdom) followed by morphological examinations. Thirty-six other fungal strains representing 36 species from 15 genera of common airborne fungi were also included in this study (Table 1). Most of these fungi

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TABLE 1. Fungal strains included in this study

<i>W. sebi</i> strains (origin)	Other species	<i>W. sebi</i> strains (origin)	Other species
UPSC 2502 (Sweden)	<i>Aspergillus niger</i> UPSC 1769	ALI 169 (China)	<i>Penicillium brevicompactum</i> ALI 319
ALI 116 (China)	<i>Aspergillus ochraceus</i> UPSC 1983	ALI 170 (China)	<i>Penicillium frequentans</i> ALI 218
ALI 117 (China)	<i>Aspergillus fumigatus</i> UPSC 1771	ALI 171 (China)	<i>Rhizopus microsporus</i> var. <i>rhizopodiformis</i> CBS 258.79
ALI 118 (China)	<i>Aspergillus flavus</i> UPSC 1768	ALI 172 (China)	<i>Rhizopus microsporus</i> UPSC 1758
ALI 119 (China)	<i>Aspergillus penicilloides</i> ALI 231	ALI 173 (China)	<i>Stachybotrys bisbyi</i> CBS 317.72
ALI 120 (China)	<i>Aspergillus versicolor</i> UPSC 2027	ALI 379 (Sweden)	<i>Stachybotrys chartarum</i> CBS 330.37
ALI 156 (China)	<i>Aspergillus silvaticus</i> ALI 234	ALI 380 (Sweden)	<i>Stachybotrys cylindraspora</i> CBS 878.68
ALI 157 (China)	<i>Cladosporium cladosporioides</i> ALI 50	ALI 381 (Sweden)	<i>Stachybotrys dichroa</i> CBS 182.80
ALI 158 (China)	<i>Chrysomya sitophila</i> ALI 346	ALI 382 (Sweden)	<i>Stachybotrys oenanthos</i> CBS 252.76
ALI 159 (China)	<i>Eurotium herbariorum</i> ALI 216	ALI 383 (Sweden)	<i>Stachybotrys kampalensis</i> CBS 388.73
ALI 160 (China)	<i>Fusarium culmorum</i> UPSC 1981	ALI 384 (Sweden)	<i>Stachybotrys microspora</i> CBS 186.79
ALI 161 (China)	<i>Gremmeniella abietina</i> ALI G144	ALI 385 (Sweden)	<i>Stachybotrys parvispora</i> CBS 253.75
ALI 162 (China)	<i>Microdochium nivale</i> UPSC 3273		<i>Saccharomyces cerevisiae</i> ALI 308
ALI 163 (China)	<i>Mucor plumbeus</i> UPSC 1492		<i>Trichoderma harzianum</i> ALI 232
ALI 164 (China)	<i>Paecilomyces lilacinus</i> UPSC 1722		<i>Trichoderma reesei</i> QM 9414
ALI 165 (China)	<i>Paecilomyces variotii</i> UPSC 1651		<i>Trichoderma viride</i> ALI 210
ALI 166 (China)	<i>Penicillium commune</i> CBS 343.51		<i>Ulocladium botrytis</i> CBS 173.82
ALI 167 (China)	<i>Penicillium italicum</i> UPSC 1577		
ALI 168 (China)	<i>Penicillium chrysogenum</i> UPSC 2020		

were obtained from the Uppsala University Culture Collection of Fungi, the Centraalbureau voor Schimmelcultures (Utrecht, Holland), and our laboratory culture collection (Arbetslivsinstitutet, Umeå, Sweden). *W. sebi* was cultivated on DG18 agar at room temperature (22°C) for 2 weeks. Other fungi were grown on 2% malt extract agar for a week at room temperature (22°C). The genomic DNAs were isolated from pure culture of each fungal strain by using a procedure described previously by Wu et al. (26). Mycelial samples of ca. 0.5 cm² were cut out from the culture plates and placed into 2-ml microtubes containing 500 µl of extraction buffer (50 mM Tris-HCl [pH 7.5], 50 mM EDTA, 2% sodium dodecyl sulfate, 1% Triton-100, 0.4 µg of RNase µl⁻¹). Two ceramic beads (4-mm diameter; Iuchi, Osaka, Japan) and 350 mg of 0.5-mm zirconia-silica beads (Biospec Products Inc., Bartlesville, Okla.) were placed into the microtube containing the mycelial sample. The tubes were placed in a Mini-Bead Beater (Biospec Products Inc.) and homogenized for 2 min at the maximum speed. The rest of the isolation procedure followed that suggested by the manufacturer of the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany).

Specific primers for *W. sebi*. Two *W. sebi* isolates from China and Sweden (ALI 158 and UPSC 2502) were sequenced for the nuclear 18S rRNA gene (25), and they had identical sequences (GenBank accession numbers AF548107 and AF548108, respectively). This sequence was aligned with the 18S rRNA sequences of the other fungal species listed in Table 1 and an additional 33 mitospore *Ascomycota* fungi accessed from GenBank (data not shown). The selection of these fungi from GenBank considered the taxonomic position of *W. sebi* in the mitospore *Ascomycota* and covered most of the major groups of mitospore *Ascomycota* available in GenBank. Unique sequence patterns of *W. sebi* were utilized to design specific primers by using the software Primer Premier version 5.0 (PREMIER Biosoftware International, Palo Alto, Calif.). Two pairs of primers were designed: Wall-SYB4 (5'-GTAGTGAACATATTTGAAGAA-3') and Wall-SYB6 (5'-ATGAGTCAATAATATAACGTC-3') (Wall-SYB4/6) and Wall-SYB7 (5'-GATTGGATGACGTTATATTAT-3') and Wall-SYB8 (5'-ACAACAAATGTCGTACCG-3') (Wall-SYB7/8). Primer pair Wall-SYB4/6 covered nucleotide positions 621 to 991 in the *W. sebi* 18S rDNA sequence (GenBank accession number AF548107), and the pair Wall-SYB7/8 covered nucleotides 963 to 1290. The expected amplicon sizes for primer pairs Wall-SYB4/6 and Wall-SYB7/8 are 371 and 328 bp, respectively. To ensure the specificity of the PCR assay, these primers were screened against sequences in GenBank by using the BLAST function (<http://www.ncbi.nlm.nih.gov/BLAST/>) to examine their possible homology to other fungi. The Search for Short, Nearly Exact Matches program was used. All four primers maintained a mismatch of >25% to any other fungal sequences in the GenBank database.

Conventional PCR and real-time PCR amplification. Conventional PCR was performed with a PTC-100 thermal cycler (MJ Research, Watertown, Mass.) in a volume of 25 µl containing 1 to 5 ng of template DNA, 10 pmol of each primer, 0.75 U of *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, Calif.), 200 µM each deoxynucleoside triphosphate (Amersham Pharmacia Biotech, Uppsala, Sweden), and 1.5 mM MgCl₂. PCR conditions were optimized to comprise an initial denaturation step of 3 min at 94°C followed by 30 cycles of

94°C for 30 s, 54°C for 30 s, and 72°C for 30 s with a final extension step of 3 min at 72°C. The PCR conditions for both sets of primers were the same. PCR products (3 µl) were analyzed by electrophoresis on 1.4% agarose gels in 1× Tris-acetate-EDTA buffer. A 1-kb Plus ladder (Invitrogen Life Technologies) was used as a DNA size standard. The gels were stained with ethidium bromide and visualized under UV light by using a Gel Doc 2000 fluorescent gel documentation system (Bio-Rad, Hercules, Calif.). A negative control and a positive control using *W. sebi* (UPSC 2502) genomic DNA were included in all PCR runs.

In real-time PCR analysis, an iQ SYBR Green Supermix kit (Bio-Rad) was used for all reactions. Real-time PCR was performed in 25 µl of a reaction volume consisting of 1 to 5 ng of template DNA, 10 pmol of each primer and 12.5 µl of iQ SYBR Green Supermix with an iCycler iQ real-time PCR detection system (Bio-Rad). The real-time PCR conditions were optimized to comprise an initial denaturation step of 3 min at 95°C followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. A melt-curve analysis immediately followed amplification at 95°C for 60 s, cooling to 60°C for 60 s, and a slow rise in temperature to 95°C at a rate of 0.5°C/10 s with continuous acquisition of fluorescence decline. Melt-curve analysis is used to observe melting characteristics of the amplicon. It is performed immediately after amplification to determine the presence of the specific product. The real-time PCR conditions for the two primer pairs were the same except that the annealing and extension times at 60°C were 60 and 30 s for Wall-SYB4/6 and Wall-SYB7/8, respectively. Each DNA sample, including the negative control, was analyzed by three replicate assays. To further confirm the specific amplification by real-time PCR, the 25 µl of PCR product was analyzed by electrophoresis on 1.4% agarose gels in 1× Tris-acetate-EDTA buffer and stained with ethidium bromide for visualization under UV light.

Sensitivity evaluation of conventional PCR and real-time PCR. To determine the detection limit of the conventional and real-time PCR, two DNA dilution series were created and subjected to PCR analyses (Table 2). The first test was done on a 10-fold dilution series of *W. sebi* genomic DNA with concentrations ranging from 1.4 to 1.4 × 10⁻⁶ ng/µl (test 1) (Table 2). The initial DNA concentration of 1.4 ng/µl was quantified by using a GeneQuant Pro RNA/DNA calculator spectrophotometer (Amersham Pharmacia Biotech). A 3-µl aliquot of each dilution (equivalent to 4.2 ~ 4.2 × 10⁻⁶ ng of DNA) was used in the PCR. To simulate the detection of *W. sebi* in a mixed fungal background, genomic DNAs of 10 common airborne fungi detected worldwide (5, 10, 14), *Aspergillus niger*, *Chrysomya sitophila*, *Cladosporium cladosporioides*, *Fusarium culmorum*, *Paecilomyces variotii*, *Penicillium commune*, *Stachybotrys bisbyi*, *Trichoderma viride*, *Eurotium herbariorum*, and *Ulocladium botrytis*, were used; each fungus had a concentration of 5 to 10 ng/µl, was mixed in an equal volume, and formed a composite fungal DNA of 7.5 ng/µl. The 10-fold dilution series of *W. sebi* DNA was mixed with this composite fungal DNA in equal volumes for conventional PCR and real-time PCR analyses (test 2) (Table 2). A 3-µl aliquot of this mixture at each dilution (equivalent to 2.1 ~ 2.1 × 10⁻⁶ ng of *W. sebi* DNA plus 11.2 ng of composite fungal DNA) was used in each PCR (Table 2).

Real-time PCR standard curve. Standard curves based on threshold cycles (at which the fluorescence signal exceeds the background during the exponential

TABLE 2. DNA dilutions and mixtures used in the sensitivity test^a

Sample no.	Test 1, <i>W. sebi</i> DNA dilution (ng/μl)	Test 2, <i>W. sebi</i> DNA dilution + fungal mix (ng/μl)
1	1.4 (4.2)	1.4 + 7.5 (2.1 + 11.2)
2	1.4×10^{-1} (4.2×10^{-1})	$1.4 \times 10^{-1} + 7.5$ ($2.1 \times 10^{-1} + 11.2$)
3	1.4×10^{-2} (4.2×10^{-2})	$1.4 \times 10^{-2} + 7.5$ ($2.1 \times 10^{-2} + 11.2$)
4	1.4×10^{-3} (4.2×10^{-3})	$1.4 \times 10^{-3} + 7.5$ ($2.1 \times 10^{-3} + 11.2$)
5	1.4×10^{-4} (4.2×10^{-4})	$1.4 \times 10^{-4} + 7.5$ ($2.1 \times 10^{-4} + 11.2$)
6	1.4×10^{-5} (4.2×10^{-5})	$1.4 \times 10^{-5} + 7.5$ ($2.1 \times 10^{-5} + 11.2$)
7	1.4×10^{-6} (4.2×10^{-6})	$1.4 \times 10^{-6} + 7.5$ ($2.1 \times 10^{-6} + 11.2$)

^a The fungal mix had a DNA concentration of 7.5 ng/μl and was composed of 10 fungi, *A. niger*, *C. sitophila*, *C. cladosporioides*, *F. culmorum*, *P. variotii*, *P. commune*, *S. bisbyi*, *T. viride*, *E. herbariorum*, and *U. botrytis*. A 3-μl aliquot was used in the PCR for test 1. A 3-μl aliquot at a 1:1 volume mix was used in each PCR for test 2. The absolute amount of DNA (ng) used in each PCR is given in parentheses.

phase of amplification) were constructed by using the 10-fold dilution series of *W. sebi* DNA (test 1) (Table 2). A 3-μl aliquot of each dilution (equivalent to $4.2 \sim 4.2 \times 10^{-6}$ ng of DNA), in three replicate assays, was used in real-time PCRs with primer pair Wall-SYB4/6 or Wall-SYB7/8. After amplification, a standard curve was automatically generated by iCycler software version 3.0a (Bio-Rad).

Bioaerosol sampling and analyses. A farm in northern Sweden was selected for the detection of *W. sebi*. The farm had a large hay and feed storage facility and a cow house. Air samples were collected during a cow feeding hour when hay and feed were handled. The airborne particles were collected and placed onto 25-mm-diameter polycarbonate filters with a pore size of 0.4 μm (Isopore; Millipore, County Cork, Ireland). The filter was mounted in a 25-mm cassette IOM sampler (SKC Inc., Dorset, United Kingdom). Air was drawn through the filter with an Aircheck Sampler model 224-PCXR7 (SKC Inc.). The airflow rate was 1.2 liters min⁻¹. The sampling time was 90 min, and about 108 liters of air was collected in each sampler. A total of six samplers were placed on the farm. The sampling flow rate and time were identical for all the six samplers. The sampling was performed during October 2003.

After sampling, the filter cassettes were brought to the laboratory for cultivation and PCR examinations. A total of 1.5 ml of suspension buffer (50 mM Tris-HCl [pH 7.5], 50 mM EDTA, 2% sodium dodecyl sulfate, 1% Triton-100) was added to each sampling cassette. The cassettes were shaken on a shaker for 10 min to suspend the particles. From each suspension, a 500-μl aliquot was serially diluted in 0.05% Tween 80. Colony counting was performed by spreading 100 μl of each dilution onto DG18 agar plates in duplicate. The DG18 plates were incubated at room temperature (22°C) for 14 days before the CFU were determined. Another 750 μl of the particle suspension was used for DNA extraction according to a method described previously by Wu et al. (24). To ensure maximum DNA recovery from each aerosol sample, DNA was eluted from the binding membrane column (DNeasy plant mini kit; QIAGEN) three times, each with 60 μl of elution buffer (buffer AE, DNeasy plant mini kit; QIAGEN). Each DNA elution was kept separately. These DNAs were analyzed by conventional and real-time PCR by using the two primer sets, Wall-SYB4/6 and Wall-SYB7/8, following the conditions described above. Through real-time PCR, the quantity of *W. sebi* DNA used as the original template in each reaction was calculated from the standard curve from which the concentration of *W. sebi* in the aerosol samples was deduced. Each of the aerosol samples and their dilutions were repeated three times in the real-time PCR analysis. Considering the complexity of bioaerosols, additional sequencing was performed on the conventional PCR-amplified products to ascertain that the PCR product from the aerosols was indeed from *W. sebi*. A sequencing reaction was performed by using the same set of primers and a BigDye Terminator cycle sequencing ready reaction kit version 3.0 (Applied Biosystems, Foster City, Calif.) and applied to an ABI 377 sequencer (Applied Biosystems). The PCR products were sequenced from both directions.

Nucleotide sequence accession numbers. The obtained sequences have been submitted to GenBank under accession numbers AY639930, AY639931, AY639932, and AY639933.

RESULTS

Specificity of the primers. As expected from the sequence data, the two primer sets Wall-SYB4/6 and Wall-SYB7/8 amplified fragments of 371 and 328 bp, respectively, in *W. sebi*

(Fig. 1A and B). All 31 *W. sebi* isolates gave identical amplification patterns, while none of the other 36 fungal species tested gave amplification products when these two primer sets were used in conventional PCR (Fig. 1A and B). The suitability of these DNAs for PCR was tested by using universal fungal primers (NS1/8 [25]) to exclude the possibility that the negative PCR was due to the absence of amplifiable DNA. In this test, all 36 fungal DNAs gave positive amplification (data not shown).

In the real-time PCR, all isolates of *W. sebi* gave strong positive fluorescent signals after 12 to 20 cycles, while the other 36 fungal DNAs produced very faint signals only after 36 cycles (panel 1 of Fig. 1C and D). Melting curve analyses indicated that the melting temperatures of the PCR products from *W. sebi* amplified with primer pairs Wall-SYB4/6 and Wall-SYB7/8 were 83.0 and 86.0°C, respectively (panel 2 of Fig. 1C and D). Both of the primer sets amplified the corresponding product in all 31 isolates of *W. sebi* as indicated by the melting curve (Fig. 1C and D). No melting peak was observed for any other fungal species, except for the weak signals observed after 36 cycles that were identified by melt-curve analysis as primer dimers. To further confirm the successful and specific real-time PCR amplification, the PCR products were further analyzed by agarose gel electrophoresis. The results indicated a clean amplification of the 371- and 328-bp fragments using Wall-SYB4/6 and Wall-SYB7/8, respectively, for all *W. sebi* isolates and negative amplification for all other fungi (data not shown). Usually, it is not necessary to have a gel test with real-time PCR. The gel test was done only to demonstrate that under the specified real-time PCR conditions, there is indeed just one amplicon of the expected size. It is possible that amplicons of a small size difference give the same melting peak. In the method development stage, all possibilities should be tested.

Detection sensitivity of conventional PCR and real-time PCR. Two dilution series of *W. sebi* DNA, with and without background fungal DNA, were created to determine the detection sensitivity of different PCR systems. Without other background DNA (test 1) (Table 2), 4.2×10^{-4} ng of *W. sebi* genomic DNA could be detected with either of the primer sets Wall-SYB4/6 or Wall-SYB7/8 (lane 5 of Fig. 2A and B, respectively) in conventional PCR. A weak amplicon could be seen from the 4.2×10^{-5} ng of DNA template (lane 6 in Fig. 2A and B). To be on the safe side, this weak signal was not considered. When *W. sebi* DNA was mixed with DNAs from 10 other fungi (test 2) (Table 2), 2.1×10^{-4} ng of *W. sebi* DNA could be detected against a background of 11.2 ng of unrelated fungal DNA (the PCR amplification profile was identical to that of test 1, shown in Fig. 2A and B; thus, the data were not shown). This finding indicates that the detection sensitivities of the two tests were of approximately the same magnitude and that the presence of other fungal background did not affect the detection of *W. sebi*. Assuming one fungal genome is ca. 4.0×10^{-5} ng of DNA (21), the conventional PCR could potentially detect 5 to 10 fungal spores in a reaction.

When the 10-fold DNA dilution series were subjected to real-time PCR, 4.2×10^{-5} ng of *W. sebi* DNA produced a distinct positive fluorescent signal (Fig. 2C and D, sample 6). With 4.2×10^{-6} ng of *W. sebi* DNA, a weak fluorescent signal was observed after 36 cycles (Fig. 2C and D, sample 7). This signal was not considered due to run-to-run variation. Thus, it

FIG. 1. Specificity examination of the primers in different PCR assays. A and B: conventional PCR amplification using the primer sets Wall-SYB4/6 and Wall-SYB7/8, respectively. M: 1-kb Plus ladder (Invitrogen Life Technologies). Fungal strains in each lane, from left to right, are in the order given in Table 1. (C and D) Panel 1: real-time PCR amplification plots with primer sets Wall-SYB4/6 and Wall-SYB7/8, respectively. The relative fluorescence units (RFU) (y axis) are plotted against PCR cycles (x axis). Panel 2: corresponding melting profiles of C and D, respectively, which display the negative first derivative of temperature versus fluorescence $[-d(RFU)/dT]$ plotted against temperature. CF, curve fit.

appears that the real-time PCR system described in this study can potentially detect one spore in a PCR. When real-time PCR was performed on the *W. sebi* DNA mixed with the composite fungal DNA (test 2) (Table 2), the amplification profile did not alter (data not shown). Thus, similar to the conventional PCR, the detection limit of the real-time PCR system was not affected by the presence of nontarget DNAs.

Detection of *W. sebi* in bioaerosols from a farm. Six aerosol samples collected from a Swedish farm were analyzed through PCR and cultivation. Three DNA elutions were collected for each sample. For most of the samples, the first and the second DNA elutions (undiluted) did not yield any amplification in the conventional PCR while the third elution did (data not shown). When the first and the second DNA elutions were diluted 100-fold, strong and specific amplification was observed in all six aerosol samples using either of the primer sets (Fig. 3A).

Similarly, in the real-time PCR assay, no fluorescent signal was observed when the first and second DNA elutions were used as a template directly, and only the third elution gave a distinct fluorescent signal (data not shown). When 1:100 dilutions of the first and second DNA eluates were used as a PCR template, strong fluorescent signals were observed in all the samples (panel 1 of Fig. 3B and C). Melting curve profiles showed that all air samples produced a characteristic single melting peak at melting temperatures of 83.0 and 86.0°C using primer sets Wall-SYB4/6 and Wall-SYB7/8, respectively (panel 2 of Fig. 3B and C), which indicated the successful detection of *W. sebi* in the aerosols.

Considering the composition complexity of bioaerosols, the PCR products from the aerosol samples were further sequenced (GenBank accession numbers AY639930, AY639931, AY639932, and AY639933) to verify their origin. Sequence analysis indicated that they matched perfectly to *W. sebi* 18S

FIG. 2. Detection sensitivity of different PCR assays. A and B: a 10-fold DNA dilution series of *W. sebi* was amplified by conventional PCR using primer sets Wall-SYB4/6 and Wall-SYB7/8, respectively. Lane 8 was the negative control. M: 1-kb Plus ladder (Invitrogen Life Technologies). C and D: real-time PCR amplification plot (relative fluorescence units [RFU] versus PCR cycles) for the DNA dilution series using Wall-SYB4/6 and Wall-SYB7/8, respectively. CF, curve fit.

rDNA (data not shown), thus demonstrating that they are indeed amplified from *W. sebi* in the aerosols.

The amount of *W. sebi* spores and hyphal fragments in the air samples was estimated from the standard curve (Fig. 4). In this study, the standard curve was generated from the 10-fold dilution series of *W. sebi* DNA as described above. Among the different PCR runs, strong linear correlations, with correlation coefficients (r^2) ranging from 1.000 to 0.995 for either of the primer sets, were maintained between log values of template DNA and real-time PCR threshold cycles over the range of DNA concentrations examined. The six aerosol samples were analyzed with both primer sets, and compatible quantification results were obtained (Fig. 4). Assuming one fungal genome is ca. 4.0×10^{-5} ng of DNA, the real-time PCR generated an estimate of 0.8×10^7 to 2.1×10^7 *W. sebi* spores m^{-3} in the aerosols, with an average value of 1.3×10^7 and 1.6×10^7 spores m^{-3} by primer sets Wall-SYB4/6 and Wall-SYB7/8, respectively (Table 3).

The six aerosol samples were also analyzed through cultivation on DG18 medium. *W. sebi* CFU were counted after 2 weeks of cultivation and revealed a concentration of 0.6×10^6 to 1.3×10^6 CFU m^{-3} , with a mean value of 0.9×10^6 CFU m^{-3} (Table 3).

DISCUSSION

Generalization on the occurrence of *W. sebi* in agricultural environments has been difficult based on the limited number of published studies. In a study involving 79 Swedish farms, the fungus was observed in 6% of the air samples (8). Another study of cattle barns in the United States concluded that *W.*

sebi does not belong to the dominant fungi (20). However, investigations using selective media favoring the growth of slowly growing fungi and inhibiting the growth of fast-growing genera all found *W. sebi* to be a predominant fungus in farms handling hay, straw, and grain and in cow barns (4, 6, 9, 16). In this study, large numbers of airborne *W. sebi* isolates were isolated in the agricultural area outside of Beijing (data not shown) and at a farm in northern Sweden. The wide distribution of *W. sebi* from Scandinavia to Asia, in some occasions at high concentrations ranging from 10^3 to 10^6 CFU m^{-3} (6, 9), suggests its common occurrence in different environments. From a public health perspective, the fungus should be investigated and monitored with more accurate detection and quantification methods.

The PCR systems developed in this study could facilitate rapid and sensitive detection of *W. sebi* in environmental samples. The two sets of PCR primers proved to be highly specific to *W. sebi* among other fungi (covering 15 genera) commonly found in aerosols of indoor and outdoor environments. The specificity and sensitivity of the detection system did not alter when applied to composite fungal DNAs in which *W. sebi* was a minority. As little as 2.1×10^{-4} ng of *W. sebi* DNA can be unambiguously detected against a background of 11.2 ng of composite fungal DNA (i.e., 1:50,000) by conventional PCR. As illustrated in Fig. 2, the detection limit of conventional PCR was not markedly lower than that of the real-time PCR as one might have expected; the latter appeared to be roughly 5- to 10-fold more sensitive. The close match in sensitivity could be partly due to the sizes of our amplicon, which are in the good detection range for agarose gel and more importantly the primer quality. As noted by other studies, the sensitivity of a

FIG. 3. Detection of *W. sebi* in aerosols from a farm in northern Sweden. A 1:100 dilution of the first DNA elution from each air sample was used as a PCR template. A: conventional PCR detection of *W. sebi* in the aerosols using primer sets Wall-SYB4/6 and Wall-SYB7/8 (panels 1 and 2, respectively). M: 1-kb Plus ladder (Invitrogen Life Technologies). Lanes 1 to 6: six aerosol samples; lane 7: negative control. (B and C) Panel 1: real-time PCR detection in the six aerosol samples; panel 2: the corresponding melting profiles using Wall-SYB4/6 and Wall-SYB7/8, respectively. CF, curve fit.

PCR assay depends on several factors, most importantly on the primer composition, structure, and homology to the target molecule (7, 19). The two sets of primers presented in this study were selected from among many pairs after intensive testing. Careful design and selection of the primers can significantly improve the sensitivity of a PCR assay. For laboratories without a real-time PCR facility, application of the conventional PCR system established in this study could still accomplish sensitive detection of *W. sebi* in environmental samples.

In the detection of *W. sebi* in aerosols from the farm, the real-time PCR gave 10-fold-higher estimates than the CFU counting. This can be explained by the presence of unviable spores and hyphal fragments in the aerosols that originated from old fungal colonies and multiple-spore aggregates that form single colonies. In DNA-based detections, all the collected bioparticles are analyzed regardless of their culturability. Thus, the real-time PCR was more sensitive in the detection and gave estimates that better reflect the true concentration of *W. sebi* in the air. Moreover, the analytical procedure was reduced to 5 to 6 h, compared to 2 weeks by cultivation on DG18 plates. The concentration of *W. sebi* (10^7 spores m^{-3}) detected on the farm by real-time PCR is similar

to the value of 3.3×10^7 *W. sebi* spores m^{-3} found in a grain elevator as revealed by direct examination of the air sampler filters by using light microscopy (4). In a Finnish study, the concentration of *W. sebi* in cow barns ranged from 10^3 to 10^6 CFU m^{-3} (6). Using the same growth medium, our cultivation revealed a level of 10^6 CFU m^{-3} on the farm. These findings suggest that *W. sebi* is common in agricultural environments and that high spore concentrations can be expected in hay and grain storage facilities and animal houses. The methods developed in this study could serve as rapid, specific, and sensitive means for the detection of *W. sebi* in aerosols and other environmental samples, thus facilitating investigations on its distribution, ecology, clinical diagnosis, and exposure risk assessment.

Two main problems are encountered when environmental samples are examined to obtain absolute quantitation: the DNA isolation efficiency and PCR inhibitors. False-negative PCRs can result from the contaminants being coextracted with DNA that acts as a PCR inhibitor. These PCR inhibitors can be removed by either further DNA purification or a dilution step (1, 3, 24). In this study, the first and second DNA elutions from the aerosols failed in PCRs, but a 1:100 dilution worked

FIG. 4. Quantification of *W. sebi* DNA in the aerosol samples through correlation to the standard curve constructed for the primer sets Wall-SYB4/6 (A) and Wall-SYB7/8 (B). y axis: threshold cycle; x axis: log starting quantity of DNA (in nanograms).

for all the PCR tests. When environmental samples are analyzed, various DNA dilutions should be tested to achieve the optimal PCR efficiency. The use of DNA dilutions to dilute out the PCR inhibitors also dilutes the DNA present; therefore, in cases where the target organism is present in low numbers, this practice may also produce a false-negative result. The implementation of an internal positive control could help to distinguish false negatives caused by dilution or inhibitors. In addition, whenever possible, further purification of the DNA sample should be recommended.

The presence of PCR inhibitors also raises the question of how the standard curve should be constructed to derive accurate quantitation for environmental samples. In most studies employing real-time PCR, including the present one, the standard curve is generated from purified DNA and used to quantify environmental samples prepared from a different matrix. For absolute quantitation, one should consider having the standard curve prepared with the matrix in question. The stan-

dard curve made from purified DNA could underestimate the real quantity in the sample. Two procedures have been suggested to correct for the matrix difference: spiking the unprocessed sample with known amounts of target spores or spiking the processed sample with known amounts of target DNA. The first approach, spiking a set of parallel unprocessed aerosol samples with a known amount of spores, would make the DNA isolation efficiency and PCR inhibitors comparable between the standard curve and the sample in the test. A limiting factor of this practice is the number of available parallel samples that can be spiked for the standard curve. In addition, the standard curve constructed in this procedure may not be linear due to different DNA isolation efficiencies at different spore concentrations (22). Spiking the processed sample with a known amount of DNA is more realistic but will not correct for the DNA isolation efficiency factor. This procedure would reveal the response of the standard curve to different amounts of PCR inhibitors, thus helping to derive better quantification for environmental samples. In general, accurate and absolute quantitation in environmental samples such as aerosols still represents a technique challenge. More experimental designs should be carried out to identify the effects of different factors on the accurate evaluation of environmental samples.

ACKNOWLEDGMENTS

This study was supported by a grant from the Swedish Council for Working Life and Social Research (FAS).

We thank the three anonymous reviewers for valuable comments and suggestions that helped to improve the manuscript.

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TABLE 3. Quantification of *W. sebi* in aerosol samples by real-time PCR and CFU counting^a

Air sample no.	CFU/m ³	Spores/m ³ by real-time PCR detection with primer set	
		Wall-SYB4/6	Wall-SYB7/8
1	1.3×10^6	2.1×10^7	1.9×10^7
2	1.1×10^6	1.1×10^7	1.4×10^7
3	0.7×10^6	0.8×10^7	1.5×10^7
4	0.8×10^6	1.0×10^7	1.7×10^7
5	1.0×10^6	1.6×10^7	1.3×10^7
6	0.6×10^6	1.0×10^7	1.5×10^7
Mean	0.9×10^6	1.3×10^7	1.6×10^7

^a Sample volume was 108 liters in all cases.

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