Quantitative Sampling Methods for the Analysis of Fungi: Air Sampling

29

Mary C. O'Loughlin, Katherine D. Turner, and Kevin M. Turner

Abstract

Quantitative sampling of fungi can be carried out in a variety of ways for a large array of purposes. These include approaches such as tape sampling, settled dust sampling, bulk material sampling, and air sampling followed by macroscopic analysis, polymerase chain reaction (PCR), or immunochemical methods for quantitation. Air sampling is widely used in a variety of industries and settings as a means of isolating material for the identification and potential enumeration of fungal strains thus we have chosen to discuss this approach to sampling in detail in this chapter. PCR overcomes the limitations of traditional culturing and macroscopic methods as it is not dependant on culturability or viability of the microorganisms. This chapter discusses a general approach to carrying out such air sampling and PCR-based quantitation.

Keywords

Quantitative sampling • Air sampling • Polymerase chain reaction

Introduction

In the nineteenth century Louis Pasteur disposed of the supposition of spontaneous generation by showing that airborne microscopic organisms account for biologic growth on previously sterile media. It has been estimated in recent times that up to 40% of homes in Northern Europe and Canada have mould contamination [1]. Various health effects, such as respiratory symptoms, allergic rhinitis, asthma, and hypersensitivity pneumonitis, are associated with mould exposure. Traditional methods for the isolation and identification of fungal spores can be time-consuming and laborious.

M.C. O'Loughlin (✉)
Department of Life Sciences, University of Limerick, Castletroy, Limerick, Ireland
e-mail: maryc.oloughlin@gmail.com

K.D. Turner
Centre for Chromosome Biology, School of Natural Sciences, National University of Ireland Galway, Galway, Ireland

K.M. Turner
Manufacturing Sciences and Technology, Pfizer Ireland Pharmaceuticals, The Pfizer Biotech Campus at Grange Castle, Clondalkin, Dublin 22, Ireland

V.K. Gupta et al. (eds.), *Laboratory Protocols in Fungal Biology: Current Methods in Fungal Biology*, Fungal Biology, DOI 10.1007/978-1-4614-2356-0_29, © Springer Science+Business Media, LLC 2013

Sampling should be performed by using validated methods and must be planned so that the smallest amount of sampling and interpretation is done to meet the information requirements. Air sampling is widely used in the detection of such moulds for eventual identification and if possible enumeration [2–5].

These air techniques are generally categorized as both passive (gravitational) and active (volumetric). Traditionally, passive air sampling (Settle plate) has been used and is still commonly in use to determine the types of microorganisms by exposing a Petri dish containing nutrient rich agar medium to the air [6, 7]. The method is somewhat criticized for being considered semiquantitative with a potential for bias towards microorganisms of larger spore size. This is primarily due to its reliance on gravity. However, it does have the potential to mimic the natural deposition of airborne spores on the surface of food products and some regard it as a dependable method to assess airborne microbial food contamination. The technique is straightforward to perform and does not involve additional investment on specialized equipment.

Active air sampling uses devices that draw a predetermined volume of air at a particular speed over a definite period of time for the assessment of viable airborne microorganisms. Though both are widely used, there has been criticism that the methodologies for sampling and analysis are neither consistent nor definitive [8]. Quantitation of sampled specimens is by and large carried out by direct microscopy, culture, or biochemical analysis but new DNA-based methods for fungal detection can now be used to enumerate the spores of fungi. Airborne spores can be collected and identified by PCR allowing identification of the species [9, 10]. However, the sample volume, short collection period, and artificial air disturbance produced by the devices may in fact affect the types and quantities of fungi captured [6]. This chapter outlines protocols for both passive and active air sampling in the context of quantitation of fungal strains from indoor areas.

Materials

All chemicals sourced from Sigma-Aldrich, St. Louis, MO, USA, unless specified.

1. SAS-super-180 sampler (PBI International, Milan, Italy).
2. Reuter Centrifugal Air Sampler (Biotest, Frankfurt, Germany) with high volume pump (Gast Inc., Benton Harbor, MI, USA).
3. Rotameter (Zefon International, Ocala, FL, USA).
4. DG18 media composed of glucose, 10 g/L; peptone, 5 g/L; NaH_2PO_4, 1 g/L; Mg_2SO_4, 0.5 g/L; dichloran, 0.002 g/L; agar,15 g/L; glycerol, 220 g/L; and chloramphenicol, 0.5 g/L.
5. Rose Bengal agar medium, MEA (Malt extract agar), CYA (Czapaek yeast extract agar), YES (Yeast extract sucrose agar), CREA (Creatine sucrose agar), and NO^2 (Nitrite sucrose agar) (Difco; Becton Dickinson, Sparks, MD, USA).
6. 9 cm Petri dish (Fisher Scientific Company, Pittsburgh, PA).
7. Parafilm-M™ (Fisher Scientific Company, Pittsburgh, PA).
8. 70% Ethanol.
9. 0.05% Tween 80.
10. 13-mm mixed cellulose ester filter 0.8 μm pore size (Fisher Scientific Company, Pittsburgh, PA).
11. Acetone-vaporizing unit (Quickfix, Environmental Monitoring Systems, Cha rleston, SC).
12. Glycerin jelly (20 g gelatin, 2.4 g phenol crystals, 60 mL glycerol, and 70 mL water).
13. 0.1% IGEPAL CA-630®.
14. Acid-washed Ballotini beads (8.5 grade, 400–455 mm in diameter) and ball mill (Glen Creston, Stanmore, UK).
15. 2× Lee and Taylor lysis buffer (100 mM Tris–HCl pH 7.4, 100 mM EDTA, 6% SDS, 2% β-mercaptoethanol).
16. Phenol:chloroform (1:1).
17. 20 μg/μL Glycogen (Roche diagnostics Ltd., Lewes, UK).

18. 6 M ammonium acetate.
19. Isopropanol.
20. TE buffer: 10 mM Tris–HCl pH 7.5 (25°C), 0.1 mM EDTA.
21. RNaseA: 10 mg/mL in TE.
22. Phenol.
23. ABI 7000 Fast Real-time PCR System (Applied Biosystems, CA, USA).
24. Universal fungal primers NS5 (5′-AA CTTAAAGGAATTGACGGAAG-3′) andNS6(5′-GCATCACAGACCTGTTATTG CCTC-3′).
25. SYBR® Premix Ex Taq™ II (×2) and ROX Reference Dye (×50) (Takara Bio., Shiga, Japan).

Methods

Active Air Sampling Using a Portable SAS-Super-180 Sampler

1. Operate the SAS-super-180 sampler at a sampling rate of 180 L air/min.
2. Follow the instruction manual provided by the producer.
3. Charge the portable battery fully prior to use.
4. Sample 500 L of air from the center of the room at a height of 1 m above the floor.
5. Use a 9-cm Petri dish containing Dichloran 18% glycerol agar medium (DG 18) [11] to trap viable fungal particles.
6. Disinfect the sampler with 70% ethanol before each use.
7. Cover the Petri dish with the lid immediately following sampling and seal with Parafilm-M™.
8. Incubate the Petri dishes containing DG18 at 25°C for 1 week in the dark and examine every 24 h.

Active Air Sampling Using a Reuter Centrifugal Air Sampler

1. Operate the Reuter centrifugal air sampler (RCS) at a flow rate of 40 L air/min [8, 12–14].
2. Follow the instruction manual provided by the producer.
3. Calibrate the high volume pump to 28.3 L/min using a rotameter.
4. Collect 15 samples of 1–15 min duration in random order at a height of 1 m above the floor.
5. Use a 9-cm Petri dish containing Rose Bengal agar medium supplemented with 100 mg/L chloramphenicol to trap viable fungal particles.
6. Disinfect the sampler with 70% ethanol before each use.
7. Cover the Petri dish with the lid immediately following sampling and seal with Parafilm-M™.
8. Incubate the Petri dishes containing Rose Bengal agar at 25°C for 1 week and examine every 24 h.

Isolation and Enumeration of Mycological Samples

1. Count the numbers of fungal colonies on each Petri dish and then subculture on Petri dishes with suitable agar media for species identification.
2. Prepare all media as per manufacturers' instructions.
3. Plate moulds belonging to *Penicillium* on the following media; MEA, CYA, YES, CREA, and NO^2.
4. Plate other moulds and yeasts on MEA and PDA (Potato dextrose agar) [11].
5. Incubate MEA, CYA, YES, and PDA in the dark at 25°C and CREA and NO2 at 20°C for 7 days [15].
6. Convert the number of CFU per plate to the number of CFU/L of air and analyze data using an appropriate statistical package.

Identification of Mycological Samples

1. Suspend individual colonies in 20 mL 0.05% Tween® 80 prepared with sterile deionized water in a test tube [5].

2. Vortex for 10 s at 20,000×*g*.
3. Filter each sample through a 0.8 μm mixed cellulose ester filter and then place on a glass slide.
4. Allow slides to dry overnight.
5. Clear the slides using a modified instant acetone-vaporizing unit
6. Mount a 25 × 25-mm cover glass on the slide using glycerin jelly.
7. Observe the slides and identify the collected fungal spores at ×400 magnification using a light microscope.

Extraction of DNA from Mycological Samples

1. Suspend individual colonies in 0.5 mL 0.1% IGEPAL CA-630® prepared with sterile deionized water in a tube.
2. Adjust the spore suspensions were adjusted to 2–3 × 10^4 spores/mL.
3. Transfer 0.4 mL of the spore suspension to a fresh tube and add 0.4 g of acid-washed Ballotini beads (8.5 grade, 400–455 mm in diameter).
4. Shake the mixture for 8 min in a ball mill.
5. Add 0.4 mL 2× Lee and Taylor lysis buffer [16].
6. Vortex the sample and incubate at 65°C for 1 h.
7. Add 0.8 mL phenol:chloroform (1:1), vortex briefly, and centrifuge at 20,000×*g* for 15 min
8. Transfer the top aqueous layer to a clean tube.
9. Add 1 μL glycogen (20 μg/μL), 40 μL 6 M ammonium acetate, and 600 μL isopropanol
10. Invert tube gently to mix and incubate at −20°C for 10 min.
11. Centrifuge at 20,000×*g* for 2 min and remove the supernatant.
12. Resuspend the pellet in 50 μL TE buffer containing RNase A (10 mg/mL) to the pellet and incubate at 37°C for 15 min.
13. Add 150 μm TE and then add 200 μL phenol
14. Vortex briefly and centrifuge at 20,000×*g* for 6 min.
15. Transfer the top aqueous layer to a clean tube.
16. Add 10 mL 6 M ammonium acetate and 600 mL isopropanol.
17. Invert tube gently to mix and incubate at −20°C for 10 min.
18. Centrifuge at 20,000×g for 10 min and remove the supernatant.
19. Add 800 mL ice cold 70% ethanol, centrifuge at 20,000×g for 2 min, and remove the supernatant.
20. Centrifuge at 20,000×g for 10 s and remove the remaining liquid.
21. Dry pellet for 20 min in a fume hood and resuspend in 50 mL TE buffer.

Quantitation of Mycological Samples Using RT-PCR

1. Use the universal fungal primer pair NS5 (5′-AACTTAAAGGAATTGACGGAAG-3′) and NS6 (5′-GCATCACAGACCTGTTATT GCCTC-3′) to amplify the 310 bp of 18S rDNA region [17].
2. Thaw the reagents and DNA preparations and keep on ice until required.
3. Dilute the extracted fungal DNA in sterile PCR-grade water to several ratios; i.e., 0.5/20, 1/20, 2/20, 4/20, and 8/20.
4. Prepare a 50-μL reaction mixture consisting of 25 μL of SYBR® Premix Ex Taq™ II (×2), 1 μL of ROX Reference Dye (×50), 2 μL of each primer (10 μM), and 20 μL of the diluted DNA extracts.
5. Program the ABI 7000 fast real-time PCR System, or equivalent system, with the following cycling conditions 95°C for 10 s, 4 cycles of 95°C for 30 s, 60°C for 31 s, and then 40 cycles of 95°C for 4 s and 60°C for 31 s.
6. Set a threshold level of 0.2 and use the auto-baseline function on the ABI 7000 software.
7. Carry out quantitative analysis of the sample using the ABI 7000 software.

References

1. Schlatter J (2004) Toxicity data relevant for hazard characterization. Toxicol Lett 153:83–89
2. Al Maghlouth A, Al Yousef Y, Al Bagieh N (2004) Qualitative and quantitative analysis of bacterial aerosols. J Contemp Dent Pract 5:91–100
3. Engelhart S, Glasmacher A, Simon A, Exner M (2007) Air sampling of Aspergillus fumigatus and other thermotolerant fungi: comparative performance of the Sartorius MD8 airport and the Merck MAS-100 portable bioaerosol sampler. Int J Hyg Environ Health 210:733–739
4. Grisoli P, Rodolfi M, Villani S, Grignani E, Cottica D, Berri A et al (2009) Assessment of airborne microorganism contamination in an industrial area characterized by an open composting facility and a wastewater treatment plant. Environ Res 109:135–142
5. Sivasubramani SK, Niemeier RT, Reponen T, Grinshpun SA (2004) Assessment of the aerosolization potential for fungal spores in moldy homes. Indoor Air 14:405–412
6. Asefa DT, Langsrud S, Gjerde RO, Kure CF, Sidhu MS, Nesbakken T et al (2009) The performance of SAS-super-180 air sampler and settle plates for assessing viable fungal particles in the air of dry-cured meat production facility. Food Control 20:997–1001
7. Scherwing C, Golin F, Guenec O, Pflanz K, Dalmaso G, Bini M et al (2007) Continuous microbiological air monitoring for aseptic filling lines. PDA J Pharm Sci Technol 61:102–109
8. Saldanha R, Manno M, Saleh M, Ewaze JO, Scott JA (2008) The influence of sampling duration on recovery of culturable fungi using the Andersen N6 and RCS bioaerosol samplers. Indoor Air 18:464–472
9. Bellanger AP, Reboux G, Murat JB, Bex V, Millon L (2009) Detection of Aspergillus fumigatus by quantitative polymerase chain reaction in air samples impacted on low-melt agar. Am J Infect Control 38:195–198
10. Lee SH, Lee HJ, Kim SJ, Lee HM, Kang H, Kim YP (2009) Identification of airborne bacterial and fungal community structures in an urban area by T-RFLP analysis and quantitative real-time PCR. Sci Total Environ 408:1349–1357
11. Pitt JI, Hocking AD (1999) Fungi and food spoilage. Aspen Publishers, Gaithersburg, MD
12. An HR, Mainelis G, Yao M (2004) Evaluation of a high-volume portable bioaerosol sampler in laboratory and field environments. Indoor Air 14:385–393
13. Yao M, Mainelis G (2007) Analysis of portable impactor performance for enumeration of viable bioaerosols. J Occup Environ Hyg 4:514–524
14. Yao M, Mainelis G (2007) Use of portable microbial samplers for estimating inhalation exposure to viable biological agents. J Expo Sci Environ Epidemiol 17:31–38
15. Amend AS, Seifert KA, Samson R, Bruns TD (2010) Indoor fungal composition is geographically patterned and more diverse in temperate zones than in the tropics. Proc Natl Acad Sci U S A 107:13748–13753
16. Lee SB, Taylor JW (1990) Isolation of DNA from fungal mycelia and single spores. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, CA, pp 282–287
17. Wu Z, Wang X-R, Blomquist G (2002) Evaluation of PCR primers and PCR conditions for specific detection of common airborne fungi. J Environ Monit 4:377–382