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ORIGINAL ARTICLE

Detection of respiratory viruses on air filters from aircraft

T.M. Korves[1], D. Johnson[2], B.W. Jones[3], J. Watson[4], D.M. Wolk[2] and G.M. Hwang[5]

1 Cognitive Tools and Data Management Department, The MITRE Corporation, Bedford, MA, USA
2 BIO5 Institute, University of Arizona, Tucson, AZ, USA
3 Department of Mechanical and Nuclear Engineering, Kansas State University, Manhattan, KS, USA
4 Office of Aerospace Medicine, Federal Aviation Administration, Washington, DC, USA
5 The MITRE Corporation, Baltimore, MD, USA

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Correspondence
Grace M. Hwang, 2275 Rolling Run Drive, Mailstop 1BS108, The MITRE Corporation, Woodlawn, MD 21244, USA.
E-mail: gmhwang@mitre.org

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Abstract

Aims: To evaluate the feasibility of identifying viruses from aircraft cabin air, we evaluated whether respiratory viruses trapped by commercial aircraft air filters can be extracted and detected using a multiplex PCR, bead-based assay.
Methods and Results: The ResPlex II assay was first tested for its ability to detect inactivated viruses applied to new filter material; all 18 applications of virus at a high concentration were detected. The ResPlex II assay was then used to test for 18 respiratory viruses on 48 used air filter samples from commercial aircraft. Three samples tested positive for viruses, and three viruses were detected: rhinovirus, influenza A and influenza B. For 33 of 48 samples, internal PCR controls performed suboptimally, suggesting sample matrix effect.
Conclusion: In some cases, influenza and rhinovirus RNA can be detected on aircraft air filters, even more than 10 days after the filters were removed from aircraft.
Significance and Impact of the Study: With protocol modifications to overcome PCR inhibition, air filter sampling and the ResPlex II assay could be used to characterize viruses in aircraft cabin air. Information about viruses in aircraft could support public health measures to reduce disease transmission within aircraft and between cities.

Introduction

Commercial air travel has a potentially important role in disseminating infectious diseases among cities and continents. Aircraft travel has hastened the spread of influenza strains (Laurel *et al.* 2001; Brownstein *et al.* 2006; Khan *et al.* 2009) and led to the intercontinental spread of severe acute respiratory syndrome (SARS) in 2002 (World Health Organization 2003). In addition, close public quarters in aircraft cabins are a concern for disease transmission (Mangili and Gendreau 2005), although documented cases of disease transmission onboard airplanes are limited (Moser *et al.* 1979; Kenyon *et al.* 1996; Olsen *et al.* 2003; Mangili and Gendreau 2005; Byrne 2007; Han *et al.* 2009; Baker *et al.* 2010). To make appropriate, cost-effective public health decisions, information about infectious viruses in aircraft is needed. Two recent studies have determined that a variety of respiratory viruses are present in symptomatic air travellers (Luna *et al.* 2007; Follin *et al.* 2009). However, at present, it is not known which viruses are typically present in aircraft cabin air, whether viruses are often present in infectious amounts (Fabian *et al.* 2008; Stelzer-Braid *et al.* 2009; Wagner *et al.* 2009; Hwang *et al.* 2011) and whether viruses survive degradation in cabin air to remain viable or detectable (Weber and Stilianakis 2008; Tang 2009). To our knowledge, only two studies have detected viruses in aircraft cabin air to date (La Duc *et al.* 2006; Yang *et al.* 2011).

One possible way to evaluate airborne viruses in aircraft is to sample aircraft ventilation filters. In a typical commercial aircraft, about half of the aircraft cabin air is recirculated after being filtered through fibreglass High Efficiency Particulate Air (HEPA) filters. These HEPA filters are expected to capture virus-sized particles with >99·9% efficiency (Bull 2008). Ventilation filters have been used to sample micro-organisms in other

environments (Echavarria *et al.* 2000; Farnsworth *et al.* 2006; Stanley *et al.* 2008). Aircraft filter material is not expected to be as efficient as other methods for collecting intact, infectious viruses because filter material may damage virus structure or damage viruses via desiccation (Mahony 2008; Verreault *et al.* 2008). Nevertheless, viral nucleic acids may remain detectable via PCR and RT-PCR. Used air filters offer a potential advantage over other sampling methods for characterizing viral diversity in aircraft because they sample air over a long period, up to 15 000 h of flight time.

To evaluate multiple viruses simultaneously (multiplex analysis), rapidly, with high sensitivity (measured as the percentage of true-positive samples that actually test positive), high specificity (measured as the percentage of true-negative samples that actually test negative) and at relatively low cost, new multiplex molecular techniques are being developed (Mahony 2008). The ResPlex II assay (QIAGEN) uses multiplex PCR and RT-PCR, coupled to bead-based flow cytometry to detect 18 different viral genetic sequences. Versions of this assay have been used to detect respiratory viruses in human samples with high sensitivity and specificity (Brunstein and Thomas 2006; Li *et al.* 2007; Wang *et al.* 2009); Li *et al.* reported sensitivity of 72–90% and specificity of 99·7–100% for six viruses in 360 clinical specimens. In this study, we evaluate the ability of the ResPlex II assay to detect inactivated viruses applied to new HEPA filters and viruses extracted from used HEPA filters from commercial aircraft.

Materials and methods

Inactivated viruses applied to new HEPA filters

To assess the ability of the assay to detect viruses on HEPA filter material, inactivated viruses were applied to 8×4 cm pieces of new HEPA filter sample that had been dry-disinfected by incubation for 30 min at 70°C; 70°C was the highest tolerable temperature recommended by a HEPA filter manufacturer. To verify that 70°C sufficiently dry-disinfected HEPA filters, filter samples after dry-disinfection were tested, and they generated subthreshold signal intensities (1–29 mean fluorescence intensity (MFI) in the ResPlex II assay described below.) New filter material was chosen instead of used filters in part because there was tremendous variability in the loading of nonbiological contaminants (e.g. lint, soot, oil) on used filters such that 'typical' filters could not be selected for testing. Furthermore, used filters may have gathered biological contaminants that could then interfere with the test results in the form of true positives or false positives. Three inactivated viruses were used: influenza A (INFA; ZeptoMetrix # NATFLUAH1-ST, lot # 303949), respiratory syncytial virus type A (RSVA; ZeptoMetrix # NATRSVA-ST, lot# 304994) and parainfluenza virus type 2 (PIV2; ZeptoMetrix # NATPARA2-ST, lot# 303911). Viruses were diluted 1 : 100 in RNAlater (Ambion, Austin, TX, USA), a reagent for stabilizing RNA, and 400 μl of each was applied to a separate HEPA filter sample. For INFA, the virus manufacturer (ZeptoMetrix, Buffalo, NY, USA) indicated that the stock solution contained approximately 7×10^8 virus copies based on quantitative PCR, which corresponds to the addition of $2{\cdot}8 \times 10^6$ influenza virus copies per filter material. For RSVA and PIV2, the virus manufacturer indicated that the initial viral stocks contained virus numbers in the range of 10^8–10^9 copies per ml. This initial viral stock range indicates that the numbers of virus copies added to each filter sample for RSVA and PIV2 were similar to that for INFA.

Because viral RNA degrades over time, the persistence of the viruses on filter material was assessed by extracting nucleic acids from filter samples at three time-points subsequent to 30-min air drying: 0, 4 and 16 h. Each time-point-virus combination was performed with two replicate samples, for a total of 18 samples. As a positive control for each time-point-virus combination, the same virus solution that was applied to the filter sample was applied directly into the ResPlex II assay in one replicate each. Additionally, in the analysis of used filter samples, inactivated viruses were also used as a positive control. For this, 133 μl of the 1 : 100 dilutions of INFA, RSVA and PIV2 were applied together to a single new filter sample; this corresponds to $9{\cdot}3 \times 10^5$ influenza virus copies and comparable amounts of the other viruses added to the sample.

Used filter samples

Used HEPA filters were removed from aircraft during maintenance checks from 23 July to 10 August 2009. Used filters had been in service for 500–15 000 h of flight time, which is approximately two to 18 months of service on aircraft. Filters came from aircraft with intercontinental routes and/or routes within North America only. Filter manufacturers and make were not identified. Filters were shipped to MITRE (McLean, VA, USA) for de-identification and then to Kansas State University (Manhattan, KS, USA) for disassembly and collection.

Care was taken to avoid contamination during collection by using gloves, masks and isopropanol-cleaned tools and surfaces, and by working in a room with filtered air. To disassemble filters, the metal frames were cut-off, and filter material was unfolded. Samples approximately 8×4 cm were cut from the centre area of the filter, with the longer dimension along the length of the pleat. Samples were cut within 24 h of receipt and shipped by next day delivery in sealed zip-lock bags to the University of

Arizona (Tucson, AZ, USA) for preservation and molecular analysis. Upon receipt, each sample was preserved in 25 ml RNAlater and stored at 4°C. The total time from filter removal from aircraft to sample storage in RNAlater ranged from 11 to 22 days. Samples were in RNAlater for 4–11·5 weeks prior to RNA extraction. One sample was analysed from each of the 48 filters.

RNA extraction

To new filter samples treated with inactivated viruses, 25 ml RNAlater was added in a 50-mL conical polypropylene tube; used filter samples were already stored in RNAlater. All samples were vortexed for 60 s. The supernatant was applied to two Amicon ultra tubes and centrifuged at 4000 ***g***, for 15 min at 4°C, to concentrate the entire volume down to approximately 400 μl. RNA was extracted according to protocols for the Qiagen EZ1 Virus Mini kit v2.0 on a Qiagen EZ1 robot in a 60-μl elution volume (Qiagen, Germantown, MD, USA). For used filter samples, three were tested for RNA concentration with a spectrophotometer, and absorption readings at 260 nm ranged from 17 to 34 ng ml^{-1} RNA.

ResPlex II assay

The ResPlex II ver. 2.0 kit (Qiagen) was used as described in the *ResPlex II Handbook*. For RT-PCR, 10 μl of extracted RNA was used per 50-μL reaction. As an internal control for amplification and detection, during RNA extraction, each sample was spiked with 60 μl of an artificial RNA fragment (ResPlex II kit) that is detectable with the ResPlex II assay. Detection was performed with a Qiagen LiquiChip 200 Workstation (Qiagen). For designating results as positive, borderline positive or negative, the MFI cut-off values suggested by Qiagen were used (http://www.qiagen.com/faq). MFI is calculated based on the number of beads that hybridize a particular PCR product; consequently, MFI values provide a semi-quantitative indication of the amount of virus present.

The ResPlex II assay includes primers and probes to detect the following viruses: adenovirus B, adenovirus E, bocavirus, coronavirus OC43, coronavirus 229E, coronavirus NL63, coronavirus HKU1, coxsackie/echovirus, human metapneumovirus, influenza A, influenza B, parainfluenza virus 1, parainfluenza virus 2, parainfluenza virus 3, parainfluenza virus 4, respiratory syncytial virus A, respiratory syncytial virus B and rhinovirus.

Statistics

The analysis of variance (ANOVA) test was calculated in R version 2.10.0 (R Development Core Team 2009).

Results

Detection of inactivated viruses applied to new high efficiency particulate air filters

For all 18 new filter samples treated with inactivated INFA, RSVA or PIV2 viruses, the ResPlex II assay yielded positive detections, with mean fluorescence intensities that ranged from 589 to 5448, all above the positive cut-off value of 250. Applied viruses were detected at all three time-points (0, 4 or 16 h) after application and drying, indicating that detectable viral RNA can persist on filter material for at least 16 h. To evaluate whether there was RNA loss owing to extraction, MFIs from the 18 test samples were compared to MFIs from positive controls, which consisted of virus directly applied to the assay without filter application and extraction. All but one of the test samples had MFI values lower than the positive control for the corresponding virus; this suggests that there was viral RNA loss during filter application and extraction. Because the ResPlex II is a semi-quantitative assay, it is not possible to derive an exact percentage for extraction loss.

Among the 18 samples, there were no false-positive results for viruses not applied. However, in three of the six samples treated with inactivated RSVA, there were elevated background signals (MFI values between 150 and 250) for a virus that was not applied, parainfluenza virus type (PIV1.) No cross-reactivity between the PIV1 and RSVA probes was previously observed in testing hundreds of clinical samples. Overall, these results show that the extraction procedure and ResPlex II assay can recover and accurately detect viruses from HEPA filter material.

Detection of viruses on used aircraft high efficiency particulate air filters

Among the 48 samples from used aircraft filters, three samples tested positive for viruses (Table 1). One was positive for INFA, one was positive for rhinovirus (RhV; most common cause of the common cold) and one was positive for both RhV and influenza B (INFB.) There were also borderline positive results for coronavirus (OC43; causes common cold), INFB and RhV (Table 1). All samples tested negative for the 14 other viruses assayed by ResPlex II.

Controls revealed no sign of virus contamination in PCR. For 15 negative controls (water), there were no positive or borderline virus results. In addition, the positive virus control (inactivated RSVA, INFA and PIV2 applied to a single new filter sample) yielded positive results for the three tested viruses (MFI values 2116, 4429 and 1461, respectively) with no positive or borderline positive results for other viruses.

Table 1 Mean fluorescence intensity results for used filter samples from aircraft

Number of used filter samples	Rhinovirus	Influenza B	Influenza A	OC43	Internal control
1	**509***	**454**	–†	235‡	**842**
1	**344**	227	–	–	**568**
1	–	–	**620**	–	**371**
5	154–224§	–	–	–	**377–539**
7	–	–	–	–	**370–744**
14	–	–	–	–	**256–364**
19	–	–	–	–	below 250

*Bold numbers are positive detections, based on the cut-off value, 250.
†'–' indicates a negative result, below the cut-off value, 150.
‡Plain text numbers indicate borderline positive results, with values in the range, 150–250.
§Numbers indicate ranges of MFI values where there is more than one sample in a group.

To assess whether amplification and detection performed properly, each sample contained an internal control consisting of an artificial transcript that can be amplified and detected by the assay. The internal control yielded MFI ranging from 371 to 842 in used filter samples that had positive or borderline positive results for viruses; these values are consistent with the assay performing as expected. Internal control values in this range (MFI ≥ 370) were seen in only seven of the 40 used filter samples that were negative for all viruses. Among the 33 samples with internal control MFI below 370, 19 had internal control MFI below the recommended 250 positive cut-off value. These weak internal control results coupled with negative virus results indicate that the assay performed suboptimally for these samples, potentially owing to the presence of PCR inhibitors. Consequently, if viruses were present, they may not have been detected.

To examine whether there was a relationship between internal control amplification and the amount of time filters were in aircraft operation, hours in service were compared for samples with MFIs above and below 370 using an ANOVA test; 370 was chosen because there were no virus detections in samples with internal control MFI below this number. The test revealed the absence of a significant association at the $P = 0{\cdot}05$ level ($F = 2{\cdot}59$ df = 1.46 $P = 0{\cdot}11$; hours in service were square root-transformed). In fact, samples with control MFI above or equal to 370 and below 370 had identical median operating times (4800 h in service) and similar ranges (≥370 range: 2100–15 190 h; <370 range: 500–10 800 h in service). In addition, there was a large range in hours in service among samples with positive and borderline detections (2730–15 190 h.) Furthermore, there was no apparent association between detection and the amount of time before sample preservation in RNAlater (range for samples with positive and borderline detections: 11–22 days, identical to the whole sample set.) Overall, 15 of 48 samples had internal control MFI above 370, and eight of these 15 samples had positive and borderline positive virus detections.

Discussion

Our results show that respiratory viral RNA is present and can be detected on some used aircraft air filters. Detection of viruses in aircraft air does not necessarily mean that infectious viruses were present; this genetic-based assay cannot distinguish between infectious and inactive virus particles, and whether influenza and other respiratory viruses have airborne transmission is controversial (Brankston *et al.* 2007; Weber and Stilianakis 2008; Tellier 2009). Nevertheless, this study suggests that the analysis of air filters with PCR-based techniques could be used as a relatively unobtrusive method to characterize airborne viruses in the aircraft cabin environment.

The presence of these viruses is not unique to air from aircraft cabins. Airborne rhinovirus has been detected in office buildings (Myatt 2004), and airborne influenza has been detected in hospital waiting rooms and clinics (Blachere *et al.* 2009; Lindsley *et al.* 2010) and a poultry market (Chen *et al.* 2009). Recently influenza virus was detected in two of three air samples taken with an impactor onboard commercial aircraft (Yang *et al.* 2011). Other viruses, varicella-zoster virus and Epstein–Barr virus (both not included in the ResPlex II assay) and coronavirus (for which there was a borderline result in our study), were also detected during flights using an impingement-based air sampler (La Duc *et al.* 2006).

It is noteworthy that viral RNA was detectable even though preservation of the samples did not occur until 11–22 days after filters were removed from aircraft. While viruses can degrade rapidly, detectable viral RNA has been

shown to persist on banknotes for more than 10 days (Thomas *et al.* 2008). More rapid preservation of filter samples might improve detection.

Despite some positive results, poor amplification of PCR internal controls in many samples indicates that the protocol did not consistently perform at optimal conditions. The ResPlex II assay and many aspects of the nucleic acid extraction procedure were designed for clinical samples, not for air samples, which are known to present two additional challenges: contaminants that inhibit PCR and low amounts of DNA and RNA (Alvarez *et al.* 1995; Blachere *et al.* 2009; Fabian *et al.* 2009). It is likely that contaminants inhibiting PCR caused the poor amplification seen for controls in many samples, as has occurred in other studies of air samples (Alvarez *et al.* 1995; Blachere *et al.* 2009; Chen *et al.* 2009). Even in samples with positive virus results, MFI values were not high, suggesting that the amounts of viral RNA in the samples may have been low. The use of other nucleic extraction methods is likely to result in improved viral RNA yield and reduced PCR inhibition. In particular, Fabian *et al.* recently reported that a Trizol–chloroform extraction method resulted in good yield and removal of PCR inhibitors for influenza virus; this paper also noted that a magnetic bead extraction method, similar to that in the QIAGEN EZ kit we used in our experiments, resulted in poor viral RNA yield (Fabian *et al.* 2009). In addition, the recently developed Synchronous Coefficient of Drag Alteration (SCODA) nucleic acid isolation method is reported to remove contaminants well (Broemeling *et al.* 2008). Finally, we note that our collaborators have consistently recovered and performed PCR on DNA from these aircraft filter samples using a modified Miller protocol (Miller *et al.* 1999) that involves phenol–chloroform–isoamyl extraction and a soil extraction kit for additional clean-up (unpublished data.)

While the limit of detection for ResPlex II assay on used filter samples is unknown, influenza virus applications of 10^6 viruses were consistently detected in our experiment, indicating that the limit of detection is below this for new filter material. The ResPlex II supplier, QIAGEN, indicates that the lower limit of detection is about 20–50 virus copies per RT-PCR (communication from QIAGEN technical support.) This implies that there would be a detection limit of 120–300 viruses per filter sample if there were no reduction owing to PCR inhibition and no loss in extraction; given the evidence for loss of RNA during extraction, a limit of detection equal to or greater than 10^3 virus particles per filter sample is likely.

Our results suggest that with improvements to nucleic acid extraction methods, the analysis of used air filter samples could be used to unobtrusively identify viruses transported by aircraft. Because air filters serve on aircraft for long periods of time, filter samples are unlikely to be used for frequent monitoring of viruses. However, the long sampling periods should make aircraft air filters especially useful for comprehensively characterizing viral background in an environment with a diverse international population. This information would provide a snapshot of infectious viruses in human populations that could inform public health efforts. In addition, this information could inform policies to reduce the spread of disease via aircraft, and technologies to reduce pathogens in aircraft. Finally, commercial aircraft is a focus of security concerns, and the ability to detect viruses in aircraft is an important step towards designing biosensors that detect and warn about dangerous viruses.

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