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Case Report

Emergence of persistent *Aspergillus terreus* colonisation in a child with cystic fibrosis

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1. Introduction

Cystic Fibrosis (CF) is the most common inherited life shortening condition affecting Caucasians. CF is characterised by mutations in the CF transmembrane conductance regulator (CFTR) gene, which codes for an ATP-driven pump that transports sodium and chloride ions across epithelial surfaces [1]. CF is a multiple organ disease; however up to 95% of morbidity and mortality is due to pulmonary infection. The CF lung has impaired mucociliary clearance and a build-up of thick mucus which creates an ideal environment to facilitate microbial colonisation. Excessive neutrophil recruitment and enhanced inflammation ensue which causes airway epithelial cell damage, decline in lung function and eventual respiratory failure.

Isolation of filamentous fungi, in particular *Aspergillus spp.* is common in respiratory secretions from CF patients [2]. *Aspergillus terreus* is the third most common filamentous fungus isolated from CF adult airway samples, being detected in 1.9 to 6.2% of CF patients [3,4]. In our clinic, 3 of 159 paediatric CF patients tested were *A. terreus* positive which is in line with the published literature on adults with CF (unpublished data). *A. terreus* has been reported to cause ABPA [5,6], infective endocarditis [6], pulmonary mycetoma [6] and invasive aspergillosis (IA) [7]. Until recently *Aspergillus* species identification was not thought to be therapeutically important; however different species within the genus can exhibit varying levels of antifungal drug resistance [8] and virulence in *in vivo* infection models [9]. Invasive disease caused by *A. terreus* can be as severe as IA caused by *Apergillus fumigatus* however *A. terreus* is inherently resistant to Amphotericin B [10]. Additionally IA caused by *A. terreus* is associated with long-term persistence of conidia and liver degeneration [11]. For these reasons *A. terreus* has the potential to cause complications post-transplant for people with CF. Here we present a case of a child with CF with a polymicrobial community in the airways among which *A. terreus* emerged and persisted as a dominant species.

2. Case

A 10-year-old boy with advanced CF lung disease diagnosed at 10 weeks old presented with a decline in pulmonary function. He had significant clinical manifestations of his disease, including chronic colonisation/infection with *Pseudomonas aeruginosa* and *Staphylococcus aureus* for more than 6 years necessitating multiple courses of antibiotic therapy, as well as gastrointestinal manifestation resulting in a body mass index (BMI) below the 0.4th centile and the requirement for gastrostomy feeding. His forced

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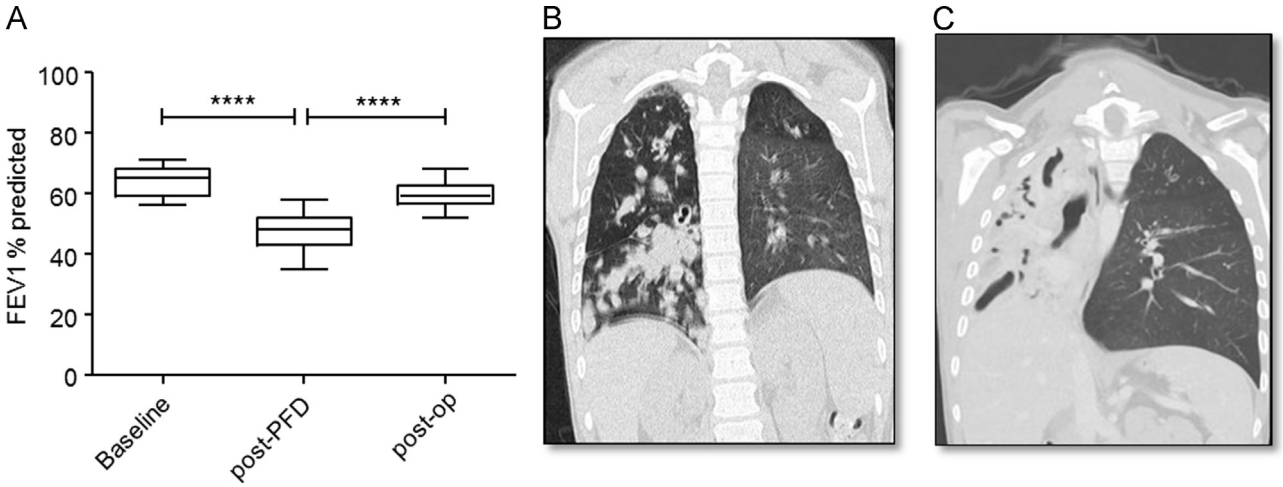

Fig. 1. Evidence of pulmonary function decline (A) Histogram depicting ranges of FEV_1 scores before initial decline in lung function (baseline), after pulmonary function decline (post-PFD) and post-pneumonectomy (post-op). Chest CT scan in month 19 (B) and in month 45 (C) revealed progressive obstruction of the right lung. $^{****}p < 0.0001$; 1-way ANOVA.

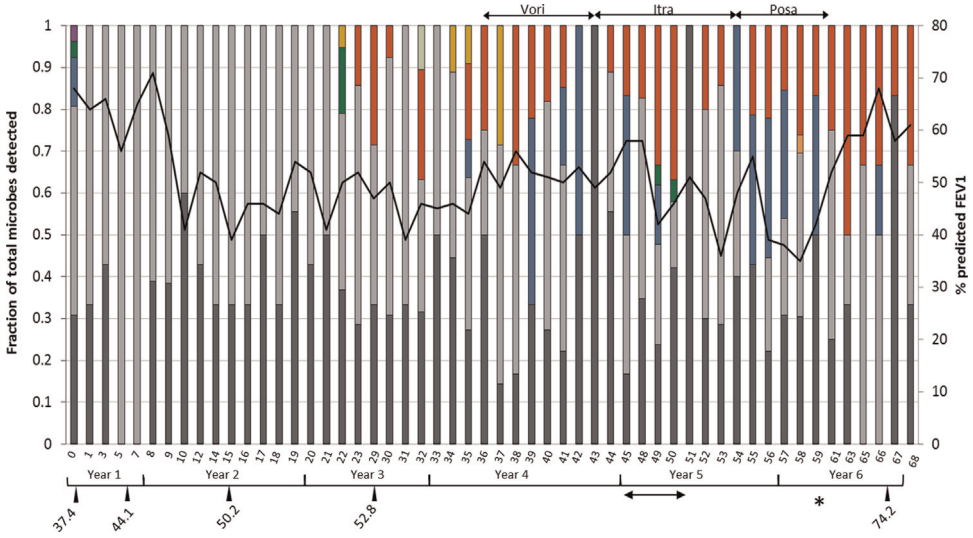

Fig. 2. Microbiological culture results and associated FEV_1 scores. A 100% stacked column histrogram presenting the microrganisms cultured from the patients airways as a fraction of the total microbial community detected by culture. *P. aeruginosa* (dark grey), *S. aureus* (light grey), *Stenotrophomonas* (blue), *A. fumigatus* (dark green), *Streptococcus pneumoniae* (purple), *Candida* (gold), *A. terreus* (red), *A. flavus* (light green) and *Escherichia coli* (orange) all colonised the airways of this patient. The secondary *y*-axis depicts the FEV_1 scores (black line) at the time of each sample collection over a six year period (*x*-axis represents months). The star (*) represents the time of right lung pneumonectomy. The doubled-ended arrow below the timeline shows the period of time that *A. terreus* was mis-identified as *P. variotii*. Periods of antifungal drug treatment are represented by doubled-ended arrows above the stacked columns; vori=voriconazole, itra=itraconazole, posa=posaconazole. Anti-*A. fumigatus* IgG levels (mg/L) are represented by drop-down arrows from the timeline. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expiratory volume in 1 s (FEV_1) was found to have significantly declined between months 8 and 10 from a score of 56–71% predicted to a score of 35–58% predicted (1 way ANOVA; $p < 0.0001$) (Fig. 1A). This coincided with an anti-*A. fumigatus* IgG level (ImmunoCap) of 44.1 mg/L (above the 40 mg/L threshold [12]) (Fig. 2), high total IgG, high total IgA and high anti-*A. fumigatus* IgE levels (ImmunoCap) (Table S1). Ten months prior to pulmonary function decline (PFD), *A. fumigatus* was detected once by culture of patient sputum on malt extract agar (Fannin) and then once again thirteen months after PFD. Fourteen months after PFD *A. terreus* was detected by culture from sputum samples. The patient subsequently remained persistently colonised with *A. terreus* (Fig. 2) and only cultured *A. fumigatus* on 2 more occasions over the 68 months (Fig. 2). The patient also tested positive for *A. flavus* on one occasion in month 32. Following six consecutive isolations of *A. terreus* from the patient's sputa, treatment with voriconazole was commenced. Due to significant side effects, this was changed to itraconazole 7 months later, and after a further 11 months this was switched to posaconazole. Susceptibility of six of the *A. terreus* isolates to the azoles was measured using the TREK Sensititre YeastOne method. Isolates were classed as resistant (R), intermediate (I) or sensitive (S) to the azoles based on published epidemiological cutoff values (ECVs) (Table S2) [13]. Despite good *in vitro* susceptibility to the azoles, *A. terreus* was not eradicated during therapy. Of note sera levels of voriconazole, itraconazole and posaconazole ranged from 0.2–1.4 mg/L, 0.34–1.44 mg/L and 0.41–0.4 mg/L, respectively (reference ranges: >2 mg/L, >0.5 mg/L and 5–15 mg/L for voriconazole, itraconazole and posaconazole, respectively). The patient's lung function continued to worsen and radiological appearances declined (Fig. 1B and C).

Twenty-two months after *A. terreus* was first identified, a fungus with a different morphological appearance was cultured on malt extract agar. In contrast to the typical cinnamon brown colonies of *A. terreus* cultured in month 40 (Fig. 3A), this isolate had

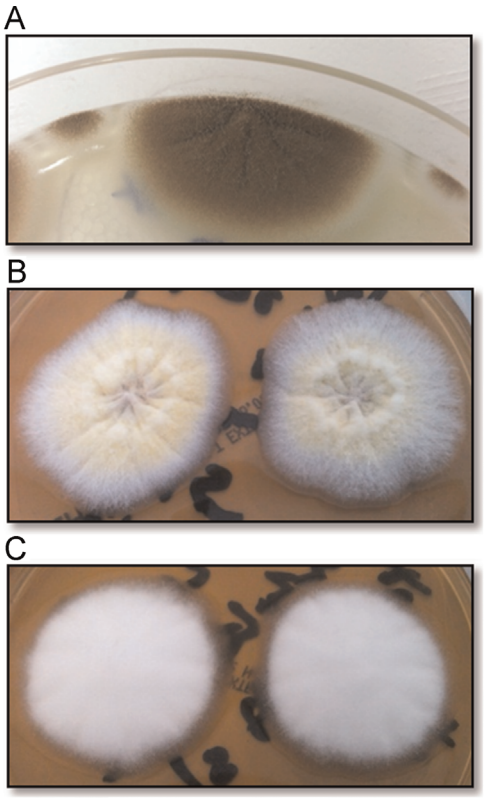

Fig. 3. Atypical growth of *A. terreus*. Typical growth of *A. terreus* collected at month 40 (A) showing cinnamon brown colonies. Two clonal variants were observed in samples plated in month 50; (B) atypical yellow sporulation (isolate 50a) and (C) white colonies with no sporulation (isolate 50b). Colonies from (A)–(C) above, along with five more isolates were sent for sequencing and all were confirmed as *A. terreus*.

yellow conidial masses (Fig. 3B) and some clonal variants grew only white hyphal masses with no spores (Fig. 3C). Microscopically these isolates had collapsed vesicles with reduced metulae and few phialides giving rise to sparse, short chains of conidia. Microscopically these isolates were similar to *Penicillium* however the yellow colour of the conidia led to their classification as *Paecilomyces variotii.* These isolates were sent for confirmatory macro- and microscopic identification and for sensitivity testing to the Health Protection Agency Mycology Reference Laboratory in Bristol, UK. These isolates were confirmed to be *A. terreus* complex, not *P. variotii*. Additionally the internal transcribed spacer (ITS), calmodulin and β-tubulin regions [14] from these isolates and five additional isolates collected from the patient were sequenced (Sourcebioscience), confirming all isolates as *A. terreus* (Table 1). Of interest the emergence of these atypical *A. terreus* isolates coincided with commencement of itraconazole therapy (Fig. 2). The

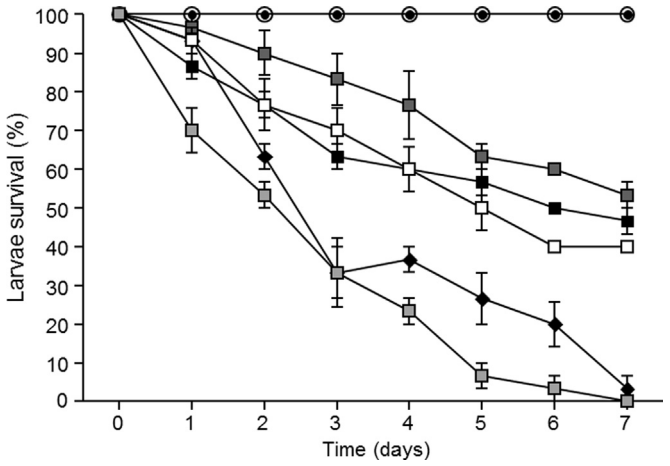

Fig. 4. Pathogenicity of *A. terreus* in the *G. mellonella* infection model. Ten Galleria were inoculated per treatment; uninjected (open circle), phosphate buffered saline (PBS) (closed circle), *A. fumigatus* reference strain (ATCC26933) (black diamond), *A. terreus* reference strain (ATCC201901) (black square) and three *A. terreus* isolates collected from the patient in month 40 (white squares), month 46 atypical isolate (light grey square) and month 50 (dark grey square). Percentage survival (*y*-axis) was monitored over 7 days (*x*-axis). These experiments were carried out on three independent occasions and error bars represent standard error.

earlier typical isolate (month 40) was sensitive to the azoles, and resistant to amphotericin B (Table 1). However the later atypical isolates (months 49 and 50) both showed intermediate resistance to voriconazole. These isolates were cultured from the patient's sputum 7 months after cessation of voriconazole therapy.

In order to establish whether the *A. terreus* isolates collected from this patient have the potential to be pathogenic, we inoculated *Galleria mellonella* larvae [15] with 1×10^6 conidia/20 μl and monitored larvae mortality over 7 days. An *A. terreus* reference strain (ATCC201901) and three *A. terreus* clinical isolates from the patient all caused mortality when tested in the *G. mellonella* infection model (Fig. 4). Interestingly one of the clinical *A. terreus* isolates with atypical growth (taken at month 46) caused 100% mortality by day 7 and was similar in virulence to *A. fumigatus* (ATCC26933).

Following complete collapse of the right lung the decision was taken to perform a right lung pneumonectomy. Post-pneumonectomy lung function returned to baseline levels however the patient continued to culture *A. terreus* and anti-*A. fumigatus* IgG levels continued to rise, with a most recent level of 74.2 mg/L. The patient was taken off posaconazole post-pneumonectomy in Newcastle in month 60 and has not been on antifungals since.

Table 1
Confirmation of eight isolates as *A. terreus* by sequencing of the ITS, calmodulin and β-tubulin regions.

Isolate no	Date isolate collected	Identification	ITS			Calmodulin			β-tubulin		
			Identity (%)	Accession no.	Query cover (%)	Identity (%)	Accession no.	Query cover (%)	Identity (%)	Accession no.	Query cover (%)
1	Month 40	*A. terreus*	99	*JQ717316.1*	100	99	*JF927626.1*	99	99	*JX501420.1*	100
2	Month 41	*A. terreus*	99	*JQ717316.1*	99	99	*LN734852.1*	100	99	*JX501420.1*	100
3	Month 44	*A. terreus*	98	*JQ717316.1*	100	99	*JF927626.1*	99	99	*JX501420.1*	100
4	Month 46	*A. terreus*	99	*JQ717316.1*	99	99	*LN734852.1*	99	99	*JX501420.1*	100
5	Month 49	*A. terreus*	99	*JQ717316.1*	100	99	*JF927626.1*	99	99	*JX501420.1*	100
6	Month 50 a	*A. terreus*	99	*JQ717316.1*	100	99	*JF927626.1*	99	99	*JX501420.1*	100
7	Month 50b	*A. terreus*	99	*JQ717316.1*	100	99	*JF927626.1*	99	99	*JX501420.1*	100
8	Month 52	*A. terreus*	99	*JQ717316.1*	100	99	*JF927625.1*	100	99	*JX501420.1*	100

3. Discussion

There are few reports of *A. terreus* colonisation of the CF airways, all of which are from adult patients [3,4]. *A. terreus* emerged as a dominant and persistent member of the polymicrobial community present in this child's airway following long-term co-colonisation with *P. aeruginosa* and *S. aureus* and a prolonged period of exposure to high-dose antibiotics (Supplementary material). Exposure to high-dose antibiotics is a predisposing factor for development of fungal infections and in particular has been linked to the development of aspergillosis in CF [16]. People with CF have a high exposure to antibiotics over their life time and as such are predisposed to developing fungal infections.

A significant decline in lung function coincided with high anti-*A. fumigatus* IgG levels of 44.1 mg/L (above the 40 mg/L threshold [12]), high total IgG, total IgA and high anti-*A. fumigatus* IgE levels but not with *A. fumigatus* or *A. terreus* culture positivity. Thirteen months after detection of anti-*A. fumigatus* IgG, *A. fumigatus* was detected in the patients sputum however this was sporadic and no *A. fumigatus* was cultured from the following 19 sputum samples. Fourteen months after detection of anti-*A. fumigatus* IgG, *A. terreus* was detected from the patients sputum and was consistently present in the patients airways thereon. It has been previously reported that serum antibody levels can be elevated in advance of sputum culture positivity for *Aspergillus* species [17]. Monitoring anti-*Aspergillus* IgG and IgE levels in vulnerable patients such as CF patients could pick up early colonisation before culture positivity. In the case presented here culture methods were used to detect microorganisms however more sensitive molecular methods may have detected *A. terreus* colonisation earlier or may have detected the presence of *A. fumigatus* in more samples. *A. fumigatus* was only cultured on 4 occasions from this patient's sputa whereas the patient was chronically colonised with *A. terreus* following initial isolation. Despite the sporadic and limited isolation of *A. fumigatus* from this patient's sputa, high *A. fumigatus*-specific IgG and IgE antibodies were detected in the patient's serum. There are no reports in the literature of cross-reactivity between *A. fumigatus* and *A. terreus* allergens using the Phadia ImmunoCap M3 assay. Phadia's online information specifies that *"A. terreus, A. flavus and A. nidulans hydrolyse collagen and were found to secrete an alkaline protease related to that of A. fumigatus*". Although the allergenic potential of this alkaline protease has not been determined it could be similar to other allergenic alkaline proteases from other fungal species and cause potential cross-reactivity with *A. terreus* using the ImmunoCap M3 assay. *A. terreus* has also been shown to produce allergens homologous to Asp f2 (UniProt: Q0CZU2_ASPTN), Asp f4 (UniProt: Q0CB03_ASPTN, Q0CTT7_ASPTN, Q0C9 × 7_ASPTN), Asp f7 (UniProt: Q0CL73_ASPTN) and Asp f15 (UniProt: Q0CYJ2_ASPTN) which could cause potential cross-reactivity as these are all identified *A. fumigatus* allergens. **The potential for cross-reactivity within the *Aspergilli* genus using the ImmunoCap M3 assay warrants further exploration.**

Lung function did not improve and radiological appearances worsened over a 4 year period despite treatment with numerous antibiotics and three azoles. Following complete collapse of the right lung the decision was taken to perform a right lung pneumonectomy. Post-pneumonectomy lung function returned to baseline levels however the patient continued to culture *A. terreus* and anti-*Aspergillus* IgG levels continued to rise, with a most recent level of 74.2 mg/L recorded in month 66. Baxter *et al.* [12] demonstrated that an *Aspergillus* IgG level of more than 75 mg/L can identify patients with ABPA serology or bronchitis with 90% sensitivity and 96% specificity. Baxter and colleagues suggest that *"the use of IgG, using a cutoff of 75 mg/L, could become more clinically useful in practice for investigating and monitoring patients clinically suspected to have Aspergillus disease"*. A study by Barton *et al.* [17] suggested a cutoff of 90 mg/L for separating patients with ABPA from those with *Aspergillus* sensitisation or controls. The patient represented in this case report had anti-*Aspergillus* IgG levels close to the 75 mg/L threshold post-pneumonectomy and with the trend of increasing anti-*Aspergillus* IgG levels observed over the past 4 years and continued culture of *A. terreus* from airway samples, the patients anti-*Aspergillus* IgG levels should be monitored closely.

Despite *in vitro* sensitivity of *A. terreus* isolates to the azoles, therapy failed to eradicate *A. terreus* from the patient's airways. It is also worth noting that there were difficulties in maintaining therapeutic levels of the azole drugs in the patient's sera. In this report later *A. terreus* isolates, post-voriconazole treatment, tested intermediate for resistance to voriconazole having been sensitive prior to this (Table S2). Here we used ECVs as there are no standardised breakpoints for antifungal drug resistance published. ECVs represent the MIC value identifying the upper limit of the wild type population and have been suggested to help characterise the susceptibility of isolates to antifungals [13]. Of note, these isolates were also independently determined as intermediately resistant to voriconazole by the Health Protection Agency Mycology Reference Laboratory in Bristol, UK (data not shown). *A. terreus* clinical isolates resistant to the triazoles have previously been reported [8,10]. These findings highlight the **need for routine antifungal drug resistance testing and in particular, monitoring of patients that receive regular high-dose azole therapy such as CF patients.**

During this study, atypical isolates of *A. terreus* were mis-identified as *P. variotii*. Previously, *A. terreus* isolates from this patient had grown with typical cinnamon brown conidial masses and the isolates mis-identified as *P. variotii* had yellow conidial masses and some clonal variants grew only white hyphal masses. Microscopically these isolates were also atypical of *A. terreus* having collapsed vesicles with reduced numbers of metulae (eliminating the circular ring of medulla typical of the species) and few phialides giving rise to sparse, short chains of conidia. Microscopically these isolates appeared similar to *Penicillium* however their yellow conidia led to their speciation as *P. variotii*. Due to this sudden appearance of *P. variotii*, a fungal species rarely isolated from CF airway samples, we sought confirmatory macro- and microscopic identification from the fungal reference laboratory in Bristol, UK and we also sequenced the ITS, β-tubulin and calmodulin regions from the atypical isolates. Also antifungal sensitivity testing was performed as *P. variotii* is susceptible to Amphotericin B [18] while *A. terreus* is intrinsically resistant to Amphotericin B. The isolates were subsequently confirmed as *A. terreus*. It is interesting that the atypical growth of *A. terreus* emerged when the patient switched from voriconazole to itraconazole therapy. Atypical growth of *Aspergillus* exposed to azoles has been previously reported to interfere with fungal identification [19]. ***A. terreus* and *P. variotii* are usually easily distinguishable by macro- and microscopic methods however the atypical growth of the *A. terreus* isolates, perhaps due to influences of azole therapy, resulted in mis-identification.** A patient's exposure to antifungals should be considered when using morphological characteristics to identify colonising fungal species.

The *A. terreus* isolates collected in this study were pathogenic in the *G. mellonella* infection model. Galleria are invertebrates with significant similarities between their immune system and the innate immune system of mammals [20]. There are also good correlations between results obtained from mice infection studies and those obtained in the galleria [21]. Interestingly, the atypical *A. terreus* isolate collected in month 46 (post-azole treatment) displayed increased virulence in the *G. mellonella* infection model. There are no reports in the literature of azoles

increasing the virulence of *A. terreus* and this warrants further investigation.

Previous studies have linked declines in pulmonary function to *Aspergillus* sensitisation [22] and *Aspergillus* colonisation in the absence of ABPA [23]. Although *A. terreus* could not be definitively linked to this patients pulmonary function decline due to the polymicrobial nature of the patient's airway, *A. terreus* could be contributing to reduced lung function and disease progression. The ultimate aim of CF care is to prevent damage to the airways caused by microbial infection and inflammation and in turn prevent lung function decline. Considering this, it is vital to establish whether rare, novel and emerging CF microorganisms are capable of impairing lung function and contributing to CF airway disease.

Conflict of interest

Dr Julie Renwick receives salary from the NCH, Tallaght hospital to carry out research. For all other authors there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mmcr.2015.07.002.

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