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A protracted course of *Pneumocystis* pneumonia in the setting of an immunosuppressed child with GMS-negative bronchoalveolar lavage

Taylor Eddens [a], Eunkyung Song [b], Monica I. Ardura [b], Jay K. Kolls [a,*]

[a] University of Pittsburgh, RK Mellon Institute for Pediatric Research, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA 15224, United States
[b] Pediatric Infectious Diseases and Immunology, Host Defense Program, Nationwide Children's Hospital and The Ohio State University, Columbus, OH 43205, United States

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ABSTRACT

We report a case of Pneumocystis pneumonia in a 5-year-old male with Trisomy 21 and acute lymphoblastic leukemia. The lack of response to trimethoprim-sulfamethoxazole raised concerns for antimicrobial resistance. Further, diagnosis of Pneumocystis in this patient was complicated by a GMS-negative bronchoalveolar lavage despite molecular evidence of Pneumocystis infection.

1. Introduction

Pneumocystis jirovecii remains a common cause of pneumonia in the immunocompromised patient population. Immunodeficient individuals, either acquired (e.g. HIV/AIDS) or genetic (e.g. severe combined immunodeficiency), are exquisitely susceptible to developing *Pneumocystis* pneumonia [1,2]. Accordingly, HIV/AIDS patients are routinely given anti-*Pneumocystis* prophylaxis, trimethoprim-sulfamethoxazole, when $CD4^+$T-cell counts fall below 200 cells/uL [3]. However, *Pneumocystis* is re-emerging in the clinical context of individuals receiving immunosuppressive drug therapy for hematologic malignancies or management of post-transplantation rejection [4]. HIV-negative patients with *Pneumocystis* tend to have a more fulminant presentation with fever, dyspnea, and chills, and have a greater extent of morbidity and mortality compared to HIV-positive patients [5]. Trimethoprim-sulfamethoxazole, in addition to use in prophylaxis, is the first line therapy for *Pneumocystis*, although treatment failure rates are greater than 20% [6].

Although the microbiology of *Pneumocystis* is difficult to elucidate due to the lack of a reliable culture method, much has been gleaned about the multiphasic life cycle of *Pneumocystis* using microscopy of the organism in the alveoli. There are two predominant forms of *Pneumocystis*: the ascus and the troph [2]. The asci are circular with a thick β-1,3-glucan shell and house eight ascospores that will be released as trophs [7]. The trophic forms are more irregularly shaped and are the replicative form of *Pneumocystis*. Reproduction occurs when two trophic forms fuse and eventually mature into an ascus. Much remains unknown about the *Pneumocystis* life *in vivo*, such as the factors that lead to sexual reproduction, trophic release, and how the balance between asci and trophs is maintained *in vivo*. GMS staining, the primary diagnostic stain used on bronchoalveolar lavage fluid, preferentially stains the asci [8]. Similarly, echinocandins, which inhibit the synthesis of β-1,3-glucan, have been shown in murine models to deplete the ascus form of infection, demonstrating that chemical modification of the *Pneumocystis* life cycle is possible *in vivo* [9].

2. Case

A 5-year-old male with Trisomy 21, Wolf-Parkinson-White syndrome, and known pre-B cell acute lymphoblastic leukemia (ALL) completing maintenance chemotherapy (with 6-mercaptopurine, methotrexate, and the final 5-day course of oral dexamethasone therapy completed 1 month prior) was hospitalized with a 2-week history of rhinorrhea, 3 days of non-productive cough, fever, and 24 h of increased work of breathing. Clinical examination on hospital day 0 revealed a child in severe respiratory distress with hypoxemia and shock requiring immediate intubation for mechanical ventilation and vasopressor support. Laboratory evaluation demonstrated leukopenia (an absolute lymphocyte count of 54 cells/mm^3 and 15% eosinophils) and thrombocytopenia (Table 1). Blood cultures were obtained and broad-spectrum antibiotics were initiated. An initial chest

* Corresponding author.

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Table 1
Laboratory results from mechanical ventilation, peripheral blood, and bronchoalveolar lavage fluid over the course of hospitalization.

	Hospital day +2	Hospital day +17	Hospital day +47
Mechanical ventilation			
Vent FIO2%	65	55	50
PEEP (cm H_2O)	16	12	8
ETCO2 (mm Hg)	42	52	Not performed
Peripheral blood			
White blood cell count (thousand/mm^3)	2.7	3.4	1.5
ALC (thousand/mm^3)	54	170	180
ANC (thousand/mm^3)	2538	2550	1200
Erythrocyte sedimentation rate (mm/h)	12	50	50
C-reactive protein (mg/dL)	8.8	0.7	15.1
LDH (U/L)	1063	3890	1383
(1,3)-β-D-glucan	Not performed	352	183
Bronchoalveolar lavage			
BAL cell count, white blood cell (WBC) count	BAL 1 WBC 64 7% lymphs	BAL 2 WBC 44 16% lymphs	Endotracheal aspirate Not performed
Pathology report	Mixed inflammation	Mixed inflammation	Mixed inflammation
Grocott's methenamine silver (GMS) stain	Positive for *Pneumocystis*	Negative for *Pneumocystis*	Negative for *Pneumocystis*
Giemsa stain	Negative for *Pneumocystis* trophozoites	Negative for *Pneumocystis* trophozoites	
Universal Fungal PCR	*P. jirovecii* DNA detected	*P. jirovecii* DNA detected	Not performed
P. jirovecii, Quantitative RT-PCR (copies/mL)	62,157,925	111,582,994	Detected, not quantifiable

radiograph revealed diffuse bilateral interstitial and alveolar infiltrates (Fig. 1(A)). Given continued hypoxemia, the patient underwent a bronchoalveolar lavage (BAL) on hospital day +2 and intravenous trimethoprim/sulfamethoxazole (T/S, 20 mg/kg/day) and systemic intravenous corticosteroids were empirically started due to concern of *Pneumocystis jirovecii* pneumonia (PCP), despite compliance with oral T/S prophylaxis. BAL histopathology revealed mixed inflammation and the Grocott's methenamine silver (GMS) stain demonstrated abundant asci of *Pneumocystis*, confirming the diagnosis of PCP (Fig. 1(B)). A real time Polymerase-Chain-Reaction (RT-PCR) assay of the BAL sample also detected *Pneumocystis jirovecii*, quantifiable at 62 million copies/mL (Focus Diagnostics, San Juan Capistrano, California).

The patient initially defervesced and became hemodynamically stable, but continued to require significant supplementary oxygen and ventilatory support including high positive end-expiratory pressures. With recrudescence of fevers, blood cultures were again obtained and empirical antimicrobials, including micafungin, were initiated. A computed tomography (Fig. 1(C)) on hospital day +17 revealed patchy airspace opacities superimposed upon diffuse ground-glass opacities bilaterally. A second BAL was performed and again revealed mixed inflammation. The GMS staining on this BAL was negative, although the qRT-PCR quantified 111 million copies/mL of *P. jirovecii* in the BAL fluid (Fig. 1(D)).

Given the lack of clinical response to T/S, the possibility of a resistant strain of *Pneumocystis* was explored. Genomic DNA from the BAL sample from hospital day +17, which was GMS-negative with rare, morphologically altered *Pneumocystis*, was isolated and the dihydropterate synthetase (*Dhps*) and the dihydrofolate reductase genes were cloned. The patient did not have any mutations within either gene that accounted for clinical non-response.

RNA from the BAL from hospital day +17 was also isolated and evaluated for *Pneumocystis* burden using qRT-PCR for the mitochondrial large subunit rRNA (LSU) (Fig. 1(E)). The patient had detectable LSU copies by qRT-PCR, corroborating the quantitative results obtained during the hospital course. However, further analyses were performed to evaluate the possible changes in *Pneumocystis* life-forms (e.g. ascus and trophozoites) [2]. β-1,3-glucan synthase (GSC-1), an ascus-enriched protein involved in fungal cell wall assembly, had decreased transcript expression in the patient by qRT-PCR compared to the LSU transcript [10]. By comparison, a positive control sample from a rhesus macaque infected with SIV and *Pneumocystis* had the opposite transcriptional profile, as GSC-1 transcript levels were substantially higher than that of LSU (Fig. 1(E)). Similar analyses were performed on the deep endotracheal aspirate collected on hospital day +47, but *Pneumocystis* RNA was not detectable at this time point.

The patient ultimately required placement of a tracheostomy tube due to prolonged intubation, and the patient received T/S for a total of 70 days. The patient was treated with a prolonged corticosteroid taper, and was ultimately able to be discharged home. An outpatient follow-up visit four months later revealed that the child was doing well, had successfully transitioned to T/S prophylaxis, had improvement in lymphopenia, and did not require additional respiratory support.

3. Discussion

PCP remains an opportunistic infection with high morbidity and mortality in immunocompromised patients [2]. With advances in cancer chemotherapy, more aggressive immunosuppressive regimens, and increasing survival, a growing proportion of patients may be at risk for PCP [11]. Breakthrough PCP in patients receiving T/S prophylaxis is unusual, but has been reported, occurring most frequently in patients with underlying hematologic malignancies [12]. When infection does occur, HIV-negative patients tend to have lower organism burdens, but have a more robust inflammatory response in the lungs than HIV-positive patients [13]. Importantly, mortality rates from PCP are higher in non-HIV patients compared with HIV-positive patients and are highest in patients with cancer, with a mortality of 36% among children with ALL [11,14,15]. Furthermore, this patient had Trisomy 21 as an underlying genetic condition, which presents its own challenges in terms of defects in

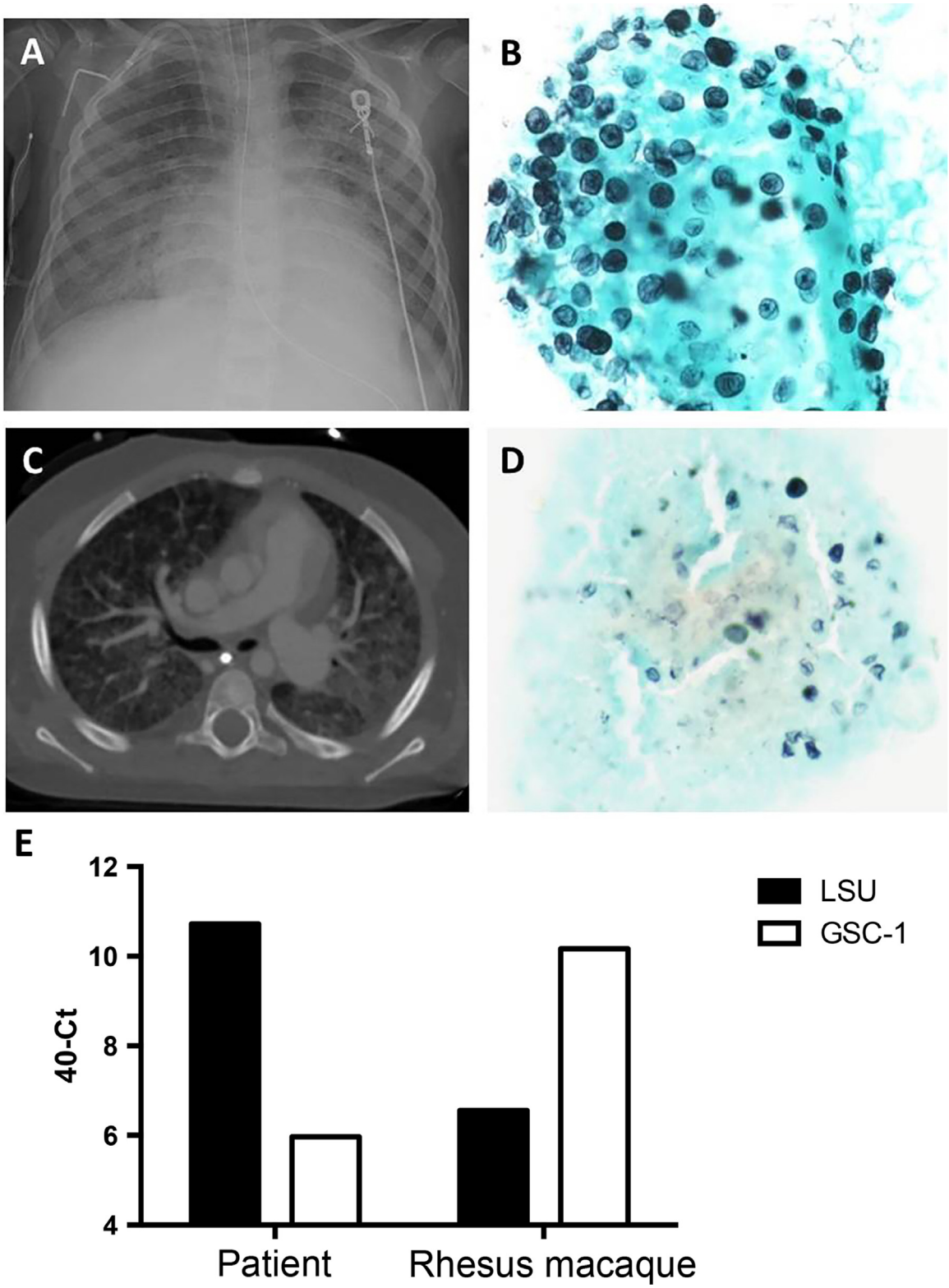

Fig. 1. Radiographic and diagnostic images over the course of hospitalization. (A) Chest radiograph from initial presentation demonstrating bilateral infiltrates. (B) GMS-positive BAL specimen obtained on hospital day +2. (C) Chest CT on hospital day +17 showing bilateral patchy airspace opacities and diffuse ground-glass opacities. (D) GMS-negative BAL on hospital day +17; second look demonstrated rare *Pneumocystis* asci with altered morphology. (E) Real-time PCR analysis on BAL fluid from the current patient and from a rhesus macaque infected with *Pneumocystis*. While the macaque had an increase in GSC-1 expression compared to LSU, the patient had decreased GSC-1 signal.

immune response and drug metabolism [16]. Such immune defects have been postulated to explain the higher incidence and severity of acute lung injury and ARDS after pneumonia compared with children without Trisomy 21 [17].

One of the biggest concerns in our patient was the possibility of T/S resistance given compliance with prophylaxis and lack of clinical response despite 2 weeks of T/S therapy. Though studies have demonstrated an association between the use of sulfa drugs

for PCP prophylaxis and *Dhps/Dhfr* gene mutations, differences in clinical outcome with *Dhps* mutations remains an area of active investigation [18]. While T/S resistance remains an important consideration on a differential diagnosis of difficult-to-treat *Pneumocystis*, the current patient was found to have no molecular evidence of decreased T/S response.

Diagnosis of PCP is largely based on the detection of *Pneumocystis jirovecii* by conventional microscopic examination and staining methods of respiratory fluids or tissue samples with GMS, as *Pneumocystis* remains unculturable. While the thick glucan cell wall of the ascus form of *Pneumocystis* will stain with GMS, the irregularly shaped trophic form will not consistently stain GMS-positive. This limitation of GMS was evident in the current patient, as the first BAL sample had an abundant number of GMS-positive, circular asci with a thick cell wall. The second BAL on hospital day +17, appeared to have scarce *Pneumocystis* asci on GMS staining. This was discordant with the PCR from the same BAL, which showed increased copies of *Pneumocystis* RNA compared to the BAL performed on hospital day +2. Without the concomitant *Pneumocystis* real-time PCR, it would have been difficult to determine if the *Pneumocystis* had resolved. One study found that a real-time PCR assay in HIV-uninfected immunocompromised hosts with clinical suspicion of PCP had a diagnostic sensitivity of 87% and specificity of 92% and a high negative predictive value [19]. While PCR assays can have utility, as in this patient, further research on the reliability and threshold of *Pneumocystis* nucleic acid levels needs to be performed.

The possible increase in *Pneumocystis* burden by real-time PCR findings in conjunction with decreasing asci observed on GMS staining also raises the interesting issue of changes in the *Pneumocystis* life cycle over the course of infection. One possible explanation is that unknown host-pathogen interactions over the course of infection favored the trophozoite life form over the ascus form. As described above, Trisomy 21 patients have immunologic defects, and as such, this patient may have had a unique niche favoring the trophozoite form. Such conditions have been determined experimentally, as pharmacologic manipulation of the life cycle of *Pneumocystis* has been well documented with the use of echinocandin class of antifungals, such as micafungin. Micafungin has been shown to inhibit synthesis of 1,3-β-D-glucan and thus selectively inhibit asci, but not trophozoites, in a rodent model of PCP [9]. While the selective effect on the asci has yet to be studied in humans, cases of progression of *Pneumocystis* have been documented while receiving echinocandins, particularly in the transplant population [20]. Therefore the utility of molecular diagnostics may have the greatest impact in patients receiving echinocandins. Ultimately, a Giemsa stain was requested on the second BAL specimen and was found to be negative for trophozoites.

The possibility of immune reconstitution inflammatory syndrome (IRIS) was also raised in this patient. However, the patient had a protracted course, not a paradoxical worsening of symptoms while receiving systemic corticosteroids. Further, there was no concrete evidence of immune recovery and microbiological improvement; in fact the patient continued to have severe lymphopenia in both peripheral blood and BAL specimens and aside from the negative GMS staining, could not explain the rising *Pneumocystis* PCR.

While micafungin therapy in the current patient was initiated simultaneously with the second GMS-negative bronchoscopy, these studies highlight the importance that different clinical scenarios may have on the *Pneumocystis* life cycle, and thus diagnosis. In our patient, the transcriptional profile was vastly different compared to an archived sample from a SIV-infected rhesus macaque and demonstrated a decrease in GSC-1, an ascus enriched protein, compared to a LSU probe. While underlying host-pathogen interactions differ between these samples, the lack of GSC-1 signal compared to LSU in our patient corroborates the reduction in asci burden by GMS. These findings highlight the importance of including *Pneumocystis* on a differential diagnosis in the presence of questionable or negative GMS, particularly if an echinocandin is included in the therapeutic regimen.

In summary, this current case of *Pneumocystis* has several unique aspects and teaching points. First, this young child with Trisomy 21 and underlying ALL had breakthrough *Pneumocystis* despite compliance with oral T/S prophylaxis and had a prolonged and severe course. Ultimately, additional molecular diagnostics allowed confirmation that the clinical specimen had no mutations conferring T/S resistance and T/S remained the therapy of choice. This patient had a negative GMS staining on BAL due to selective pressure on the ascus form of *Pneumocystis*, further highlighting the importance of understanding the plasticity of the *Pneumocystis* life cycle and selectivity of diagnostic stains. This case exemplifies the necessity of considering *Pneumocystis* in the differential diagnosis in an immunocompromised patient requiring respiratory support, even in the setting of a GMS-negative bronchoalveolar lavage.

Conflict of interest

There are none.

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References

[1] K. Djawe, K. Buchacz, L. Hsu, M.J. Chen, R.M. Selik, C. Rose, et al., Mortality risk after AIDS-defining opportunistic illness among HIV-infected persons-San Francisco, 1981–2012, J. Infect. Dis. (2015).
[2] T. Eddens, J.K. Kolls, Pathological and protective immunity to Pneumocystis infection, Semin. Immunopathol. 37 (2) (2015) 153–162.
[3] A. Morris, J.D. Lundgren, H. Masur, P.D. Walzer, D.L. Hanson, T. Frederick, et al., Current epidemiology of Pneumocystis pneumonia, Emerg. Infect. Dis. 10 (10) (2004) 1713–1720.
[4] R. Maini, K.L. Henderson, E.A. Sheridan, T. Lamagni, G. Nichols, V. Delpech, et al., Increasing Pneumocystis pneumonia, England, UK, 2000–2010, Emerg. Infect. Dis. 19 (3) (2013) 386–392.
[5] S. Mori, M. Sugimoto, *Pneumocystis jirovecii* infection: an emerging threat to patients with rheumatoid arthritis, Rheumatology 51 (12) (2012) 2120–2130.
[6] J. Helweg-Larsen, T. Benfield, C. Atzori, R.F. Miller, Clinical efficacy of first- and second-line treatments for HIV-associated *Pneumocystis jirovecii* pneumonia: a tri-centre cohort study, J. Antimicrob. Chemother. 64 (6) (2009) 1282–1290.
[7] C.M. Aliouat-Denis, A. Martinez, E.L.M. Aliouat, M. Pottier, N. Gantois, E. Dei-Cas, The Pneumocystis life cycle, Mem. Inst. Oswaldo Cruz 104 (3) (2009) 419–426.
[8] C.F. Thomas, A.H. Limper, Pneumocystis pneumonia, N. Engl. J. Med. 350 (24) (2004) 2487–2498.
[9] M.T. Cushion, M.J. Linke, A. Ashbaugh, T. Sesterhenn, M.S. Collins, K. Lynch, et al., Echinocandin treatment of pneumocystis pneumonia in rodent models depletes cysts leaving trophic burdens that cannot transmit the infection, PLoS One 5 (1) (2010) e8524.
[10] T.J. Kottom, A.H. Limper, Cell wall assembly by Pneumocystis carinii. Evidence for a unique gsc-1 subunit mediating beta-1,3-glucan deposition, J. Biol. Chem. 275 (51) (2000) 40628–40634.
[11] A. Roux, E. Canet, S. Valade, F. Gangneux-Robert, S. Hamane, A. Lafabrie, et al., *Pneumocystis jirovecii* pneumonia in patients with or without AIDS, France, Emerg. Infect. Dis. 20 (9) (2014) 1490–1497.
[12] P. Fillatre, , O. Decaux, S. Jouneau, M. Revest, A. Gacouin, F. Robert-Gangneux, Incidence of Pneumocystis jiroveci pneumonia among groups at risk in HIV-

negative patients, Am. J. Med. 127 (12) (2014) 1242.e11–1242.e17.
[13] E. Catherinot, F. Lanternier, M.E. Bougnoux, M. Lecuit, L.J. Couderc, O. Lortholary, *Pneumocystis jirovecii* Pneumonia, Infect. Dis. Clin. North Am. 24 (1) (2010) 107–138.
[14] X. Monnet, E. Vidal-Petiot, D. Osman, O. Hamzaoui, A. Durrbach, C. Goujard, et al., Critical care management and outcome of severe Pneumocystis pneumonia in patients with and without HIV infection, Crit. Care 12 (1) (2008) R28.
[15] W.T. Hughes, R.A. Price, H.K. Kim, T.P. Coburn, D. Grigsby, S. Feldman, Pneumocystis carinii pneumonitis in children with malignancies, J. Pediatr. 82 (3) (1973) 404–415.
[16] J.Y. Alaama, M.S. Ahmad, S. Ahmad, Z.A. Damanhouri, Altered metabolism in down syndrome, in: S. Dey (Ed.), Health Problems in Down Syndrome, InTech, Croatia, 2015.
[17] M. Bruijn, L.B. van der Aa, R.R. van Rijn, A.P. Bos, J.B. van Woensel, High incidence of acute lung injury in children with Down syndrome, Intensiv. Care Med. 33 (12) (2007) 2179–2182.
[18] K. Crothers, C.B. Beard, J. Turner, G. Groner, M. Fox, A. Morris, et al., Severity and outcome of HIV-associated Pneumocystis pneumonia containing *Pneumocystis jirovecii* dihydropteroate synthase gene mutations, Aids 19 (8) (2005) 801–805.
[19] E. Azoulay, A. Bergeron, S. Chevret, N. Bele, B. Schlemmer, J. Menotti, Polymerase chain reaction for diagnosing pneumocystis pneumonia in non-HIV immunocompromised patients with pulmonary infiltrates, Chest 135 (3) (2009) 655–661.
[20] R. Utili, E. Durante-Mangoni, C. Basilico, A. Mattei, E. Ragone, P. Grossi, Efficacy of caspofungin Addition to Trimethoprim-sulfamethoxazole Treatment for Severe Pneumocystis Pneumonia in Solid Organ Transplant recipients, 2007 Sep 27.