METAGENOMIC PROFILING OF POTENTIAL PATHOGENS ISOLATED FROM BLOOD SAMPLES AND TRACHEAL ASPIRATES OF CRITICALLY ILL CHILDREN WITH PERSISTENT FEVER UNRESPONSIVE TO BROAD-SPECTRUM ANTIBIOTICS

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Abstract. Critically ill children with refractory fever despite broad-spectrum antimicrobial therapy are common encountered in infectious disease. Causes of persistent fever in these cases are rarely identified by conventional diagnostic methods. Clinical metagenomics provides a promising approach to identify known and novel pathogens when currently available methods fail to yield diagnosis. Potential pathogens in blood and/or tracheal aspirates from critically were identified from 34 critically ill children <15 years of age with refractory fever despite receiving broad-spectrum antibiotics using a metagenomic shotgun sequencing platform. Multiple microorganisms were identified in 82% of blood samples, with *Stenotrophomonas maltophilia* and *Mycobacterium chelonae* being the two most common bacteria (62% each), followed by *Pseudomonas aeruginosa* (9%). The three most common viruses identified in blood were herpes simplex virus/HSV (26%), rhinovirus (20%), and human bocavirus (HBoV) (9%). Distribution of organisms in tracheal aspirates was comparable to that in blood samples. In conclusion, the metagenomic technique allowed identification of both potential pathogenic bacteria and viruses in blood and trachea of critically ill children with refractory fever and receiving broad-spectrum antibiotics; however, these findings should be interpreted with caution as no data from a case control group were available.

Keywords: *Mycobacterium chelonae*, *Stenotrophomas maltophilia,* children, critically ill, fever, metagenomics, pneumonia, respiratory failure.

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INTRODUCTION

One of the frequently asked questions during infectious disease consultation is the cause of persistent fever among critically ill patients despite broad spectrum antibiotic treatment and negative or non-contributory laboratory results (Rehman and deBoisblanc, 2014). Each year, approximately 200-300 children are admitted in the pediatric intensive care unit (PICU) of Queen Sirikit National Institute of Child Health, Bangkok, Thailand due to severe lower respiratory infection with respiratory failure (Vongpakorn, 2009). These patients constitute a highly susceptible population with disproportionately high incidence of nosocomial infection due to invasive procedures, drug resistance and patients' weak defense mechanism (Valles and Ferrer, 2009), with many failing to improve despite the use of available antiviral and/or broad-spectrum antibiotics. In a large proportion of these cases, the causative pathogen cannot be identified using conventional bacterial culture system and/or molecular diagnostics technology for viral pathogens (Chun *et al*, 2015).

In order to prevent excessive and unnecessary use of antimicrobial/antiviral agents in these patients, it is essential to distinguish between systemic inflammatory response syndrome (SIRS) and sepsis. Furthermore, pathogen identification as a cause of sepsis will enable use of proper antimicrobials. Although conventional blood cultures are considered gold standards for pathogen identification, various factors may result in low diagnostic yield, such as presence of nonbacterial pathogens, a low level of bacteremia, prior exposure to antibiotics, presence of fastidious or intracellular bacteria, and improper sample collection (*eg* insufficient amount of blood sampled) (He *et al*, 2006; Tiwari *et al*, 2013). In addition, these techniques require dedicated specialized staff and suffer from their intrinsic inadequacy in limiting multiplication of a fastidious organism, including several major viruses. Thus, polymerase chain reaction (PCR)-based detection techniques, flow cytometry and mass spectrometry have gained importance for their direct detection of pathogens with a shorter turnaround time (Hunfeld *et al*, 2008; Klouche and SchrÖder, 2008). The advantages of these techniques, particularly PCR, are numerous in terms of speed, cost, automation, sensitivity, and specificity. However, the major disadvantage of a pathogen-specific PCR is that it is only able to identify predefined targets and its lack of genotype specificity, particular for highly variable or multiple genotype viruses such as enteroviruses or adenoviruses (Epsy *et al*, 2006).

At the same time, substantial progress has been made in DNA sequencing technology, allowing large numbers of DNA samples to be rapidly sequenced (Grumaz *et al*, 2020). The availability of next generation sequencing (NGS) technologies has the potential to transform and revolutionize infectious disease diagnostics and discoveries. "Clinical metagenomics" has the ability to analyze whole microbial communities from clinical samples based on huge data obtained from metagenomic shotgun sequencing, resulting in a rapidly-growing field of clinical metagenomics that provides a new insight into the relationship between the host and microbes (Chiu and Miller, 2019). Adoption of this technology in clinical practice remains a challenge owing to cost and limited ability to handle a large data set, which requires a bioinformatics specialist in data interpretation.

Several case reports and clinical studies have demonstrated the value of NGS as a single diagnostic tool where traditional diagnostics have otherwise failed; NGS has the potential to be broadly applicable to infectious disease diagnostics (Chiu and Miller, 2019). For example, it is estimated 63% of encephalitis cases are left without an identified etiologic agent despite extensive testing (Glaser *et al*, 2006). Existing case reports have shown successful adoption of NGS technology to diagnose unusual, emerging, or rare causative etiologies for encephalitis, such as *Leptospira santarosai* (Wilson *et al*, 2014), astrovirus (Naccache *et al*, 2015) and bornavirus (Hoffmann *et al*, 2015). In a study of 40 different diagnostic tests, which were employed but failed to yield any causes, a single NGS assay is successful in identifying the treatable pathogen(s) (Wilson *et al*, 2014). Similarly, utilization of NGS in identifying divergent astrovirus clades in a pair of encephalitis cases highlighted the value of this technique in elucidating the disease spectrum of this virus and its zoonotic potential (Quan *et al*, 2010).

Similar to PCR technology, NGS offers a major way forward in microbial diagnostics beneficial in clinical practice. In April 2015, the American Academy of Microbiology conducted a colloquium to critically evaluate the trends in the use of NGS for infectious disease diagnostics (American Academy of Microbiology, 2016). Despite its vast potential, one of the major challenges in clinical application is the handling the enormous volume of data produced by NGS. One particular limitation involves differentiating organisms that are "innocent bystanders" or colonizers from true pathogens.

Here, a study was conducted using metagenomic shotgun next generation sequencing to determine its diagnostic performance in identifying causative pathogen(s) causing persistent fever among critically ill children despite broad spectrum antibiotic treatment and negative or noncontributory laboratory results. The finding may be helpful to inform the potential etiologies and guide proper intervention such as antimicrobial option for this vulnerable critically ill population.

MATERIALS AND METHODS

Patients' enrollment

An observational descriptive study was conducted at the pediatric intensive care unit (PICU), Queen Sirikit National Institute of Child Health (QSNICH), Bangkok, Thailand. Inclusion criteria were: i) children 1 month to 15 years of age receiving care at PICU, QSNICH with radiographic confirmed pneumonia and respiratory failure (both community-acquired and nosocomial pneumonia); and ii) presence of fever with axillary temperature ≥38°C for at least 48 hours after receiving broad-spectrum antibiotics, *eg* carbapenem with or without other antibiotics (beta-lactam plus beta-lactamase inhibitors, colistin, quinolones, tigecycline, or vancomycin). Clinical and outcome characteristics were obtained from medical records. Results from blood and tracheal aspirate culture or viral isolation by other methods according to routine investigation were obtained and compared to those from NGS findings.

The study protocol was approved by the Ethics Committee of Queen Sirikit National Institute of Child Health (EC approval no. 048/2558). Prior written informed consent was obtained from parents or legally guardian of each enrolled subject.

Sample collection

A 3 ml aliquot of EDTA-blood sample was obtained with aseptic precaution and then centrifuged at 2,000 *g* for 10 minutes and plasma and blood were stored at -50°C until used. In addition, 3-5 mL aliquot of tracheal aspirate was obtained from the same patient and divided into two parts: i) a small amount of the aspirate was transferred to a sterile flocked swab and submerged in 3 ml of universal transport medium (Copan UTM®) and stored at -50°C and ii) the remaining sample was also stored at -50°C until used. All samples were then sent for DNA extraction and sample preparation at the Medical Genetics Center, Medical Life Science Institute, Department of Medical Sciences, Ministry of Public Health, Thailand before submitted to NGS at the Research Institute for Microbial Diseases (RIMD), Osaka University, Japan.

DNA/RNA extraction, Illumina library preparation and sequencing

Plasma and tracheal aspirate samples were suspended in TRIzol reagent (Invitrogen, Carlsbad, CA) and RNA was then extracted using a PureLink RNA Mini Kit (Ambion, Austin, TX). DNA was extracted from blood samples using a DNeasy PowerSoil Kit (QIAGEN, Hidlden, Germany). Each RNA sample was reverse transcribed with SuperScript III Reverse Transcriptase (Invitrogen) using an N9 random primer (Takara,Kusatsu, Shiga, Japan) and cDNA synthesized using Klenow (exo-)polymerase (New England Biolabs, Ipswich, MA). Metagenomic shotgun libraries were prepared with a Nextera XT Kit (Illumina) and sequenced (100 bp) using a HiSeq 2500 platform (Illumina).

Similarity search and taxonomic assignment

Illumina adapter sequences were removed using cutadapt-1.2.1 program (National Bioinformatics Infrastructure Sweden) and "trimmed" sequences were mapped to a human reference sequence (hg19) using Stampy software (Wellcome Centre for Human Genetics, Oxford, UK). Sequences not mapped to the human genome unmapped were used subjected to BLAST search of NCBI-nt (https://www.ncbi.nlm.nih.gov/genbank/) with a $\log_e$ value cutoff of -5. Taxonomic information of each sequence was assigned and relative abundance of each detected organism was determined by text mining of BLASTn output files using BioRuby scripts (BioRuby: Open-source bioinformatics library).

Bioinformatics analysis

The results obtained from NGS generally consisted of several genetic fragments belonging to different types of bacteria, viruses, fungi, and protozoa. Therefore, a careful interpretation is necessary to decide which pathogen(s) is (are) the most likely cause of refractory fever. In the present study, NGS results are reported based on the top five highest reads of organisms known or have been previously reported as causing human infection (IOM Forum on Microbial Threats, 2009).

Statistical analysis

Data were analyzed descriptively using a Statistical Package for Social Sciences software version 15.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

From February 2015 to February 2016, 34 patients at PICU, QSNICH were enrolled. Approximately three-fourth of cases were children under 5 years of age. Seventy-three percent of cases had underlying disease of which cardiovascular abnormalities being the most common followed by respiratory and genetic or congenital abnormalities. In addition to pneumonia and respiratory failure, common co-morbidities included septic shock with and without acute respiratory distress syndrome (Table 1). The overall case fatality rate was 23% (*n* = 8) and median [interquartile range (IQR)] length of stay 27 (16-43) days. The proportion of positive viral pathogens identified in blood samples by NGS was high (75%) among fatality cases compared to patients who survived (46%). Nevertheless, owing to the limited number of patients, it was not possible to identify independent risk factors for fatality or differentiate the effect of antibiotic use on survival outcome as all study subjects received relatively similar broad-spectrum antibiotic, such as carbapenem, colistin or vancomycin during their admission.

According to standard conventional and routine investigations, only one case (3%) had positive blood culture

Table 1
Demographic profile of participants at the pediatric intensive care unit, Queen Sirikit National Institute of Child Health, Bangkok, Thailand (February 2015 - February 2016).

Characteristic	Number (%) (*n* = 34)
Male sex	18 (53)
Mean age	3 years
1 months-<1 year	10 (29)
1-5 years	15 (44)
6-10 years	7 (21)
11-15 years	2 (6)
Presence of underlying diseases	25 (73)
Cardiovascular system	8 (23)
Respiratory system	7 (21)
Genetics or congenital abnormality	6 (18)
Malignancy	5 (15)
Neurological system	4 (12)
Presence of co-morbidity/other diagnosis	16 (47)
Septic shock	6 (18)
Septic shock with ARDS	2 (6)
Meningoencephalitis	2 (6)
Varicella zoster infection	1 (3)
Myocarditis	1 (3)
Kawasaki's disease	1 (3)
Tracheitis	1 (3)
IAHS	1 (3)
Dengue Infection	1 (2.9)
Mean duration of admission prior to specimen collection	6 days

ARDS: Acute respiratory distress syndrome for *Pseudomonas stutzeri*, while 59% of cases had positive potential pathogens identified in tracheal aspirates, with 15 different types of bacteria (11 with single species and 4 with multiple species) (Table 2). Three cases had positive fungal cultures in tracheal aspirates (one case with an unidentified yeast and two cases with *Candida albicans*). There were two cases with a single virus infection identified by conventional respiratory panel PCR in tracheal aspirates (one case of respiratory syncytial virus and one case of rhino/enterovirus). There were three cases with positive identification of both bacteria and viruses in tracheal aspirates (rhino/enterovirus with *Moraxella catarrhalis*, parainfluenza with *Acinetobacter baumannii*, and rhino/enterovirus with *A. baumannii*) and three cases with positive identification of both bacteria and fungi (two cases with *C. albicans* with *A. baumannii* and one case of *Flavobacterium* spp with an unidentified yeast). In tracheal aspirates the most common potential pathogen was *A. baumannii* (53%), followed by *Pseudomonas aeruginosa* (13%), *and Stenotrophomas*

Table 2
Comparative analysis of potential bacterial pathogens identified in blood and tracheal samples between conventional culture and next generation sequencing (NGS) techniques.

Bacterial pathogen	Blood sample (n = 34)		Tracheal aspirate (n = 34)	
	Culture *n* (%)	NGS *n* (%)	Culture *n* (%)	NGS *n* (%)
Gram-positive bacteria	0	1 (3)	1 (3)	1 (3)
Streptococcus pneumoniae	0	1 (3)	0	0
Staphylococcus aureus	0	0	1 (3)	1 (3)
Gram-negative bacteria	0	31 (91)	14 (41)	39 (115)*
Stenotrophomonas maltophilia	0	21 (62)	2 (6)	23 (67)
Acinetobacter baumanii	0	2 (6)	8 (23)	6 (18)
Pseudomonas aeruginosa	0	3 (9)	2 (6)	4 (12)
Pseudomonas stutzeri	1 (3)	3 (9)	0	1 (3)
Klebsiella pneumoniae	0	2 (6)	0	0
Enterobacter cloacae	0	0	0	1 (3)
Aeromonas hydrophila	0	0	0	1 (3)
Serratia spp	0	0	0	1 (3)
Elizabethkingia meningosepticum	0	0	0	1 (3)
Flavobacteriaceae spp	0	0	2 (6)	1 (3)
Mycoplasma pneumoniae	0	1 (3)	0	4 (12)
Nocardioides spp JS614	0	1 (3)	0	0
Mycobacterium spp	0	14 (41)	0	12 (35)
M. chelonae	0	12 (35)	0	12 (35)
M. abscessus	0	2 (6)	0	0
Multiple potential pathogens	0	28 (82)	4 (12)	25 (73)

*Due to multiple pathogens in the same sample.

maltophilia (13%).

NGS technique, as expected, identified more potential bacterial pathogens in blood and tracheal aspirate samples than from cultures (Table 2). From blood samples, the most common potential pathogens identified using NGS were Gram-negative bacilli, in particular *S. maltophilia* (62%). Of note, NGS technique also identified genetic elements of other groups of bacteria, *eg Mycobacterium chelonae* (62%) and *Mycoplasma pneumoniae* (3%). There were 28 cases with multiple potential pathogens in blood samples. From tracheal aspirates, NGS technique identified more Gram-negative bacterial species than in blood samples, with *S. maltophilia* being the most common (68%). A lower number of samples with *M. chelonae* and higher number with *Myc. pneumoniae* were detected in tracheal aspirates than in blood. There were 25 cases with multiple potential pathogens in in tracheal aspirates. Of the 34 episodes of refractory fever, 21 (62%) of the episodes demonstrated concordance between potential bacterial pathogens identified in blood samples and tracheal aspirates based on NGS analysis. The two most common potential bacterial pathogens identified in these two types of samples were *S. maltophilia* and *M. chelonae*. In addition, there were 3 (9%) cases where *Aspergillus oryzae* was identified in both blood samples and tracheal aspirates.

Tracheal aspirates of eight cases (23.5%) were sent for multiplex PCR for 18 strains of a respiratory virus panel based on the physician's discretion as a part of routine investigation during the early phase of PICU, QSNICH admission. Rhinovirus/enterovirus was the most commonly identified pathogen (50%), followed by parainfluenza type 3 and respiratory syncytial virus (37.5% each).

NGS technique identified 17 different types of viruses in blood samples and tracheal aspirates (Table 3). In blood samples the most common viruses detected were rhinovirus C (12%), followed by human bocavirus (HBoV), herpes simplex virus (HSV1), human herpes virus 7 (HHV7), rhinovirus A and respirartory syncytial virus (RSV) (9% each). In tracheal aspirates HBoV was the most common virus (18%) followed by parainfluenza virus and rhinovirus C (12% each). From individual case analysis, rhinovirus C (*n* = 2), HBoV (*n* = 1), HSV (*n* = 1), and RSV (*n* = 1), were identified in both blood sample and tracheal aspirate from the same individual suggesting the invasive nature and potential cause of refractory fever in these patients. NGS assay also identified several unusual viral pathogens, *eg* GB virus C in blood sample of two cases and human coronavirus HKU1 in tracheal aspirate of one case. Among the eight cases whose tracheal aspirates were sent for conventional multiplex PCR of respiratory viruses, five (62.5%) cases demonstrated concordance with NGS findings. The most common virus identified by both methods was rhino/enterovirus (*n* = 2) and parainfluenza type 3 (*n*= 2) followed by RSV (*n* = 1), and HBoV (*n*= 1). One case had concurrently positive for 3 viruses for both multiplex PCR and NGS, *ie* HBoV, parainfluenza type 3 and RSV. No potential viral pathogens were identified among two cases by both multiplex PCR and NGS methods.

DISCUSSION

The study clearly demonstrates the superiority of NGS assay over conventional culture technique in detecting potential pathogenic pathogens in blood samples and tracheal aspirates as

Table 3
Distribution of viral genetic fragments identified in blood and tracheal aspirates by next generation sequencing.

Viral pathogen	Blood (*n* = 34) *n* (%)	Tracheal aspirate (*n* = 34) *n* (%)
Herpes simplex virus 1 (HSV1)*	3 (9)	2 (6)
Epstein-Barr viras (EBV)	2 (6)	0
Cytomegalo virus (CMV)	1 (3)	1 (3)
Human herpes virus 7 (HHV7)	3 (9)	0
Rhinovirus A	3 (9)	1 (3)
Rhinovirus B	0	1 (3)
Rhinovirus C*	4 (12)	4 (12)
Enterovirus D	1 (3)	1 (3)
Human respiratory syncytial virus*	3 (9)	3 (9)
Human Metapneumovirus	0	1 (3)
Human Parainfluenza virus	0	4 (12)
Human Bocavirus*	3 (9)	6 (18)
Influenza A	0	1 (3)
Influenza B	0	1 (3)
hepatitis G virus C	2 (6)	0
Human coronavirus HKU1 (HCoV-HKU1)	0	1 (3)
Human coronavirus NL63 (HCoV-NL63)	0	1 (3)
Multiple viruses	1 (3)	7 (20)

*Identified concomitantly in blood and tracheal aspirates in the same individual (*n* = 5, 15%).

causes of refractory fever among critically ill children with severe pneumonia requiring mechanical ventilation. With its high sensitivity, multiple types and species of micro-organisms were concurrently identified; many of them had not be reported to be associated with human disease or pathogenic condition.

S. maltophilia was the most predominant potential pathogen identified in both blood samples and tracheal aspirates by NGS. This potential pathogen is recognized as a global multidrug-resistant opportunistic pathogen commonly associated with respiratory infection in healthcare environment (Yu *et al*, 2016). This finding may be attributed to its intrinsic resistance to several antibiotic classes via beta-lactamase production, efflux pumps and reduced membrane permeability (Brooke, 2012). Furthermore, resistance phenotypes are known to change during the course of treatment, complicating interpretation of the initial antibiogram profile (Garrison *et al*, 1996).

Despite being recognized as a nosocomial pathogen, the precise modes of transmission of *S. maltophilia* in health care settings remain uncertain (Gherardi *et al*, 2015). Important features of this pathogen include ubiquitous presence, ability to persist in nutrient-poor aqueous environment and to form biofilms (An and Berg, 2018). Thus, *S. maltophili* can adhere to various types of materials, *viz* intravenous cannula, nebulizers and prosthetic devices, thereby causing nosocomial infection (Brooke, 2012). In addition, a metagenomics analysis of hospital air identified *Stenotrophomonas*, *Aspergillus* and *Penicillium* spp. among the most abundant pathogens (King *et al*, 2016).

Although *S. maltophilia* can be detected in several samples using NGS method , very few cases were identified by conventional blood and tracheal aspirate culture method. This could be attributed, in part, to *S. maltophilia* being generally associated with polymicrobial infections or growing slowly in the host, resulting in difficulty in culturing this bacterium. The use of selective media such as Gram-negative selective agar medium or medium containing antimicrobial agents such as vancomycin, imipenem, and amphotericin B (VIA medium) has been recommended (Denton *et al*, 2000; Moore *et al*, 2003).

M. chelonae was among the most commonly identified potential pathogens in blood samples and tracheal aspirates. This organism belongs to the group of rapidly growing mycobacteria (RGM), which are ubiquitous environmental organisms and are mostly resistant to traditional anti-tuberculous agents (Jones *et al,* 2019). Bloodstream infection due to RGM is an emerging problem and poses a therapeutic challenge (Rodriguez-Coste *et al*, 2016). The majority of nosocomial *M. chelonae* infection and other RGM occur in a population with multiple comorbidities, mostly immunosuppression and malignancy; in addition, they are associated with catheter-associated sepsis (McCracken *et al*, 2000; Antony, 2015; Mankad *et al*, 2015;). In more severe cases, this can turn into mycobacterial endocarditis (Yuan *et al*, 2015). A retrospective review of cases of nontuberculous mycobacteria (NTM) in children indicated *M. chelonae/abscessus* are predominant strains in the pediatric population (Iroh Tam *et al*, 2015).

Another mycobacterium identified in blood samples was *M. abscessus*, which is attributed as a cause of catheter-related bloodstream infection (Su *et al*, 2013), outbreak in PICU (Jamal *et al*, 2014) and bacteremia in compromised hosts (Lee *et al*, 2014). The role of these pathogens as a cause of refractory fever or SIRS needs to be confirmed in future studies that include control groups.

Mycoplasma pneumoniae was identified in 12% tracheal aspirates. In general, mycoplasma pneumonia is considered a community-acquired infection. However, a few reports have identified this organism as a causative agent of nosocomial pneumonia (Wang *et al*, 2010). This may be due to its relatively long incubation period (1-4 weeks). It is possible that some patients may have already been infected with this organism before admission and the infection subsequently becomes evident. Nilsson *et al* (2008) demonstrated persistence of *Myc. pneumoniae* DNA in airways with median time of carriage of 7 weeks (range 2 days to 7 months) after disease onset. Thus, it is possible that the detection of *Myc. pneumoniae* could also be the result of a recent infection rather than an active one. Nevertheless, *Myc. pneumoniae* can cause nosocomial pneumonia following assisted ventilation, suggesting hospital transmission of this pathogen (Casalta *et al*, 1996). On the other hand, a high proportion of asymptomatic children carry *M. pneumoniae* in the upper respiratory tract and current molecular diagnostic technology or serology cannot discriminate between *Myc. pneumoniae* infection and carriage (Meyer Sauteur *et al*, 2016). In addition, clinical and radiographic characteristics are rather nonspecific for *Myc. pneumoniae* infection

(Meyer Sauteur *et al*, 2016).

Blood culture provided very little information on the cause of refractory fever. Only one case exhibited a positive blood culture for *P. stutzeri*, while three samples were positive by NGS. *P. stutzeri* is a saprophytic Gram-negative, nonfermenting rod bacterium found in soil and water (Halabi *et al*, 2019). *P. stutzeri* has been reported to be present in wound infection, community-acquired pneumonia, bacteremia, osteomyelitis, septic arthritis, eye infections, otitis media, community-acquired pneumonia, brain abscess, and bacteremia mainly among immunosuppressed patients (Goetz *et al*, 1983; Keys *et al*, 1983; Puzenat *et al*, 2004; Yee-Guardino *et al*, 2006; Bello, 2007). The etiological role of *P. stutzeri* in refractory fever or SIRS remains to be confirmed because infections caused by this organism are rare and it is usually considered a saprophytic organism.

The most common viral genetic elements identified in blood samples were from human Herpes virus group. Whether this finding indicated these viruses were the cause of fever or evidence of viral reactivation during the critically ill period was not clear. Berrington *et al* (2009), in a retrospective analysis of blood samples from hospitalized patients, found sepsis and respiratory failure in 31 and 23% of cases respectively with detectable HSV DNA in cases with relatively higher mortality rate of 60% in immunocompromised hosts and 33% in immunocompetent patients. Lepiller *et al* (2015), from examination clinical relevance of HSV viremia in critically ill patients in ICUs, reported HSV DNA in 11.8% of cases, the majority of whom with HSV-viremia episodes have immunosuppressed status or are on mechanical ventilation (79.5 and 65.9% respectively). HSV disseminated infection has been reported mainly in neonates and/or severely immunosuppressed children (Holzinger *et al*, 2010; Nagamori *et al*, 2012; Kotzbauer *et al*, 2014; Maeba *et al*, 2015; Patrick *et al*, 2015).

The most common respiratory virus detected in blood samples was human rhinovirus/enterovirus (HRV/ENT), followed by RSV and HBoV, in keeping with a report from Spain HBoV is the third most common virus, after RSV and rhinovirus, in severe respiratory infection among children ≤14 years of age (Garcia-Garcia *et al*, 2007).

HBoV was the most common potential viral pathogen identified in tracheal aspirates, followed by HRV/ENT, parainfluenza virus, and RSV. In individual case analysis, HBoV, RSV, and rhinovirus C were identified in both blood sample and tracheal aspirate from the same individual, suggesting the invasive nature and potential cause of refractory fever in these patients.

One case of HCoV-HKU1 was identified in tracheal aspirate. This virus was first identified in Hong Kong in January 2005 (Woo *et al*, 2005) and is associated with mostly self-limiting upper and lower respiratory infection (Lau *et al*, 2006; Woo *et al*, 2009). A survey in Korea in 2007-2008 identified HCoV-HKU1 in 2.5% of respiratory samples from patients with acute respiratory illness (Lee *et al*, 2013).

Two cases were positive for GB Virus C using NGS assay. This virus, formerly known as hepatitis G virus (GBV)-C/HGV, is a positive, single-stranded flavivirus (Giret and Kallas, 2012). Although transmission via the blood-borne route is the most common mode, vertical and sexual transmission has also been documented (Wiwanitkit, 2006). GB Virus C is distributed globally with 10-fold higher prevalence in African compared to non-African countries, but its clinical significance in human infection remains unclear (Sathar *et al*, 2000; Abe, 2001; Waqar *et al*, 2002; George and Varmaz, 2005). Approximately 12-15% of chronic non-A, B and C hepatitis cases are infected with GB Virus C (Sathar *et al,* 2000), but a direct association with liver pathology is still lacking and it is unclear whether GB Virus C is indeed a hepatotropic virus.

One major consideration of NGS diagnostics in clinical practice is the lack of standardization or consensus on the minimum microbial/human DNA ratio resulting in clinical conditions (including infection and/or inflammation or increased cancer risk) (de Leng *et al*, 2016). This value is likely to be species-specific: for example, in order to identify the potential cause of Merkel cell carcinoma, Feng *et al* (2008) established a viral/human RNA of 0.0005% as being a value associated with carcinogenesis, while Palacios *et al* (2008) considered a viral/human RNA of 0.0135% for a new arenavirus to be indicative of the cause of fatality in undifferentiated febrile illness.

Molecular diagnosis has been successful in confirming the discovery of an emerging pathogen as the cause of severe acute respiratory syndrome (SARS)-coronavirus outbreak in 2003 (Chan *et al*, 2004). The same virus was identified in various specimens, such as stool, gastric content, and pooled nasal and throat swabs, suggestive of extensive systemic replication, which correlated with disease severity. Thus, to make a strong conclusion regarding a causative organism, it may be helpful if the same/similar potential pathogen can be identified in multiple body fluid specimens, especially when investigating and/or making diagnosis of emerging infectious diseases.

Another important concern is the reliability of samples not being contaminated from environmental sources. This issue requires stringent containment and analysis protocols when using NGS technology in identifying potential pathogens in clinical samples. Laurence *et al* (2014) and Strong *et al* (2014) reported bacterial contamination is an important issue requiring extensive consideration in NGS-based pathogen detection and diagnosis as the amount of bacterial contamination can be high enough to impact diagnosis. In addition, other types of contamination in the NGS process have been reported (Naccache *et al*, 2013; Percudani, 2013), although mitigation methods have been proposed (Cibulskis *et al*, 2011; Schmieder and Edwards, 2011; Jun *et al*, 2012). Thus, results from NGS techniques should be confirmed by other independent methods such as RT-PCR and PCR (Laurence *et al*, 2014; Strong *et al*, 2014). Interpretation of results remains difficult for most technicians, and even more for clinical microbiologists. Better guidelines are needed for interpretation of the results in order to fully utilize NGS technology in clinical diagnosis.

The study suffers from a major weakness: a lack of case controls to allow interpretation of the results, in particular the viruses detected by NGS technique and not previously found in such patients. Some of these potential pathogenic agents may be ubiquitous in blood and lungs of patients with compromised lung function.

In conclusion, notwithstanding these reservations, NGS technology holds promise as a sensitive and specific tool for identification of pathogens among critically ill children (and others) with severe pneumonia with refractory fever. However, suitable control samples from healthy subjects and/or paired samples obtained after recovery, will be required in future studies.

ACKNOWLEDGEMENTS

The research was supported in part by a Japan-Thai bilateral research protect from JSPS and National Research Council of Thailand .

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

Abe K. GB virus-C/hepatitis G virus. *Jpn J Infect Dis* 2001; 54: 55-63.

American Society for Microbiology. Applications of clinical microbial next-generation sequencing: report on an American Academy of Microbiology Colloquium; 2015 Apr. Washington DC: American Society for Microbiology; 2016 [Cited 2020 Apr 12]. Available from URL: https://www.ncbi.nlm.nih.gov/books/NBK513764/

An SQ, Berg G. *Stenotrophomonas maltophilia*. *Trends Microbiol* 2018; 26: 637-8.

Antony SJ. Catheter related line sepsis resulting from *Mycobacterium chelonae* infection in an immunocompromised host. *Infect Disord Drug Targets* 2015; 15: 135-7.

Bello CS. *Pseudomonas stutzeri*: a rare cause of neonatal septicaemia. *East Mediterr Health J* 2007; 13: 731-4.

Berrington WR, Jerome KR, Cook L, Wald A, Corey L, Casper C. Clinical correlates of herpes simplex virus viremia among hospitalized adults. *Clin Infect Dis* 2009; 49: 1295-301.

Brooke JS. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clin Microbiol Rev* 2012; 25: 2-41.

Casalta JP, Piquet P, Alazia M, Guidon-Attali C, Drancourt M, Raoult D. *Mycoplasma pneumoniae* pneumonia following assisted ventilation. *Am J Med* 1996; 101: 165-9.

Chan PK, To WK, Ng KC, *et al*. Laboratory diagnosis of SARS. *Emerg Infect Dis* 2004; 10: 825-31.

Chiu CY, Miller SA. Clinical metagenomics. *Nat Rev Genet* 2019; 20: 341-55.

Chun K, Syndergaard C, Damas C, *et al*. Sepsis pathogen identification. *J Lab Autom* 2015; 20: 539-61.

Cibulskis K, McKenna A, Fennell T, Banks E, DePristo M, Getz G. ContEst: estimating cross-contamination of human samples in next-generation sequencing data. *Bioinformatics* 2011; 27: 2601-2.

de Leng WW, Gadellaa-van Hooijdonk CG, Barendregt-Smouter FA, *et al*. Targeted next generation sequencing as a reliable diagnostic assay for the detection of somatic mutations in tumours using minimal DNA amounts from formalin fixed paraffin embedded material. *PLoS One* 2016; 11: e0149405.

Denton M, Hall MJ, Todd NJ, Kerr KG, Littlewood JM. Improved isolation of *Stenotrophomonas maltophilia* from the sputa of patients with cystic fibrosis using a selective medium. *Clin Microbiol Infect* 2000; 6: 397-8.

Espy MJ, Uhl JR, Sloan LM, *et al*. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin Microbiol Rev* 2006; 19: 165-256.

Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science* 2008; 319: 1096-100.

Garcia-Garcia ML, Calvo Rey C, Pozo Sanchez F, *et al*. Human bocavirus infections in Spanish 0-14 year-old: clinical and epidemiological characteristics of an emerging respiratory virus. *An Pediatr* (Barc) 2007; 67: 212-9. [in Spanish]

Garrison MW, Anderson DE, Campbell DM, *et al*. *Stenotrophomonas maltophilia*: emergence of multidrug-resistant strains during therapy and in an *in vitro* pharmacodynamic chamber model. *Antimicrob Agents Chemother* 1996; 40: 2859-64.

George SL, Varmaz D. What you need to know about GB virus C. *Curr Gastroenterol Rep* 2005; 7: 54-62.

Gherardi G, Creti R, Pompilio A, Di Bonaventura G. An overview of various typing methods for clinical epidemiology of the emerging pathogen *Stenotrophomonas maltophilia*. *Diagn Microbiol Infect Dis* 2015; 81: 219-26.

Giret MT, Kallas EG. GBV-C: state of the art and future prospects. *Curr HIV/AIDS Rep* 2012; 9: 26-33.

Glaser CA, Honarmand S, Anderson LJ, *et al*. Beyond viruses: clinical profiles and etiologies associated with encephalitis. *Clin Infect Dis* 2006; 43: 1565-77.

Goetz A, Yu VL, Hanchett JE, Rihs JD. *Pseudomonas stutzeri* bacteremia associated with hemodialysis. *Arch Intern Med* 1983; 143: 1909-12.

Grumaz C, Hoffmann A, Vainshtein Y, *et al*. Rapid next-generation sequencing-based diagnostics of bacteremia in septic patients. *J Mol Diagn* 2020; 22:405-18.

Halabi Z, Mocadie M, El Zein S, Kanj SS. *Pseudomonas stutzeri* prosthetic valve endocarditis: a case report and review of the literature. *J Infect Public Health* 2019; 12: 434-7.

He F, Zhang X, Zhou J, Liu Z. A new MSPQC system for rapid detection of pathogens in clinical samples. *J Microbiol Methods* 2006; 66: 56-62.

Hoffmann B, Tappe D, Hoper D, *et al*. A variegated squirrel bornavirus associated with fatal human encephalitis. *N Engl J Med* 2015; 373: 154-62.

Holzinger D, Kuhn J, Ehlert K, Groll AH. HSV-1 viremia as a potential cause of febrile neutropenia in an immunocompromised child. *J Pediatr Hematol Oncol* 2010; 32: e19-21.

Hunfeld KP, Bingold T, Brade V, Wissing H. Molecular biological detection of pathogens in patients with sepsis. Potentials, limitations and perspectives. *Anaesthesist* 2008; 57: 326-37. [in German]

Institute of Medicine (IOM) Forum on Microbial Threats. Infectious disease emergence: past, present, and future. In: IOM (US), editor. Microbial evolution and co-adaptation: a tribute to the life and scientific legacies of Joshua Lederberg: Workshop Summary. Washington DC: National Academies Press (US); 2009. Also available from: URL: https://www.ncbi.nlm.nih.gov/books/NBK45714/

Iroh Tam PY, Kline S, Ward G, Ferrieri P. Non-tuberculous mycobacterial infection in hospitalized children: a case series. *Epidemiol Infect* 2015; 143: 3173-81.

Jamal W, Salama MF, Al Hashem G, *et al*. An outbreak of *Mycobacterium abscessus* infection in a pediatric intensive care unit in Kuwait. *Pediatr Infect Dis J* 2014; 33: e67-70.

Jones RS, Shier KL, Master RN, Bao JR, Clark RB. Current significance of the *Mycobacterium chelonae-abscessus* group. *Diagn Microbiol Infect Dis* 2019; 94: 248-54.

Jun G, Flickinger M, Hetrick KN, *et al.* Detecting and estimating contamination of human DNA samples in sequencing and array-based genotype data. *Am J Hum Genet* 2012; 91: 839-48.

Keys TF, Melton 3rd LJ, Maker MD, Ilstrup DM. A suspected hospital outbreak of pseudobacteremia due to *Pseudomonas stutzeri. J Infect Dis* 1983; 147: 489-93.

King P, Pham LK, Waltz S, *et al*. Longitudinal metagenomic analysis of hospital air identifies clinically relevant microbes. *PLoS One* 2016; 11: e0160124.

Klouche M, SchrÖder U. Rapid methods for diagnosis of bloodstream infections. *Clin Chem Lab Med* 2008; 46: 888-908.

Kotzbauer D, Frank G, Dong W, Shore S. Clinical and laboratory characteristics of disseminated herpes simplex virus infection in neonates. *Hosp Pediatr* 2014; 4: 167-71.

Lau SK, Woo PC, Yip CC, *et al*. Coronavirus HKU1 and other coronavirus infections in Hong Kong. *J Clin Microbiol* 2006; 44: 2063-71.

Laurence M, Hatzis C, Brash DE. Common contaminants in next-generation sequencing that hinder discovery of low-abundance microbes. *PLoS One* 2014; 9: e97876.

Lee MR, Ko JC, Liang SK, Lee SW, Yen DH, Hsueh PR. Bacteraemia caused by *Mycobacterium abscessus* subsp *abscessus* and *M. abscessus* subsp *bolletii*: clinical features and susceptibilities of the isolates. *Int J Antimicrob Agents* 2014; 43: 438-41.

Lee WJ, Chung YS, Yoon HS, Kang C, Kim K. Prevalence and molecular epidemiology of human coronavirus HKU1 in patients with acute respiratory illness. *J Med Virol* 2013; 85: 309-14.

Lepiller Q, Sueur C, Solis M, *et al*. Clinical relevance of herpes simplex virus viremia in Intensive Care Unit patients. *J Infect* 2015; 71: 93-100.

Maeba S, Hasegawa S, Shimomura M, *et al*. Successful treatment of corticosteroid with antiviral therapy for a neonatal liver failure with disseminated herpes simplex virus infection. *AJP Rep* 2015; 5: e089-092.

Mankad S, Karthik R, Rupali P, Michael JS. Fatal disseminated *Mycobacterium chelonae* infection in an immunocompromised host--a unique presentation. *J Assoc Physicians India* 2015; 63: 49-52.

McCracken D, Flanagan P, Hill D, Hosein I. Cluster of cases of *Mycobacterium chelonae* bacteraemia. *Eur J Clin Microbiol Infect Dis* 2000; 19: 43-6.

Meyer Sauteur PM, Unger WW, Nadal D, Berger C, Vink C, van Rossum AM. Infection with and carriage of *Mycoplasma pneumoniae* in children. *Front Microbiol* 2016; 7: 329.

Moore JE, Xu J, Millar BC, Courtney J, Elborn JS. Development of a Gram-negative selective agar (GNSA) for the detection of Gram-negative microflora in sputa in patients with cystic fibrosis. *J Appl Microbiol* 2003; 95: 160-6.

Naccache SN, Greninger AL, Lee D, *et al*. The perils of pathogen discovery: origin of a novel parvovirus-like hybrid genome traced to nucleic acid extraction spin columns. *J Virol* 2013; 87: 11966-77.

Naccache SN, Peggs KS, Mattes FM, *et al*. Diagnosis of neuroinvasive astrovirus infection in an immunocompromised adult with encephalitis by unbiased next-generation sequencing. *Clin Infect Dis* 2015; 60: 919-23.

Nagamori T, Koyano S, Asai Y, *et al*. Sequential changes in pathophysiology of systemic inflammatory response in a disseminated neonatal herpes simplex virus (HSV) infection. *J Clin Virol* 2012; 53: 265-7.

Nilsson AC, Bjorkman P, Persson K. Polymerase chain reaction is superior to serology for the diagnosis of acute *Mycoplasma pneumoniae* infection and reveals a high rate of persistent infection. *BMC Microbiol* 2008; 8: 93.

Palacios G, Druce J, Du L, *et al*. A new arenavirus in a cluster of fatal transplant-associated diseases. *N Engl J Med* 2008; 358: 991-8.

Patrick K, Ali M, Richardson SE, *et al*. The yield of monitoring for HSV and VZV viremia in pediatric hematopoietic stem cell transplant patients. *Pediatr Transplant* 2015; 19: 640-4.

Percudani R. A microbial metagenome (*Leucobacter* sp.) in *Caenorhabditis* whole genome sequences. *Bioinform Biol Insights* 2013; 7: 55-72.

Puzenat E, Chirouze C, Khayat N, *et al*. Ecthyma gangrenosum caused by *Pseudomonas stutzeri* with bacteraemia and systemic vascularitis. *Rev Med Interne* 2004; 25: 315-8. [in French]

Quan PL, Wagner TA, Briese T, *et al*. Astrovirus encephalitis in boy with X-linked agammaglobulinemia. *Emerg Infect Dis* 2010; 16: 918-25.

Rehman T, deBoisblanc BP. Persistent fever in the ICU. *Chest* 2014;145:158-65.

Rodriguez-Coste MA, Chirca I, Steed LL, Salgado CD. Epidemiology of rapidly growing mycobacteria bloodstream infections. *Am J Med Sci* 2016; 351: 253-8.

Sathar M, Soni P, York D. GB virus C/hepatitis G virus (GBV-C/HGV): still looking for a disease. *Int J Exp Pathol* 2000; 81: 305-22.

Schmieder R, Edwards R. Fast identification and removal of sequence contamination from genomic and metagenomic datasets. *PLoS One* 2011; 6, e17288.

Strong MJ, Xu G, Morici L, *et al*. Microbial contamination in next generation sequencing: implications for sequence-based analysis of clinical samples. *PLoS Pathog* 2014; 10: e1004437.

Su SH, Chen YH, Tsai TY, *et al*. Catheter-related *Mycobacterium abscessus* bacteremia manifested with skin nodules, pneumonia, and mediastinal lymphadenopathy. *Kaohsiung J Med Sci* 2013; 29: 50-4.

Tiwari DK, Golia S, K TS, C LV. A study on the bacteriological profile and antibiogram of bacteremia in children below 10 years in a tertiary care hospital in Bangalore, India. *J Clin Diagn Res* 2013; 7: 2732-5.

Valles J, Ferrer R. Bloodstream infection in the ICU. *Infect Dis Clin North Am* 2009; 23: 557-69.

Vongpakorn J. Acute lung injury and acute respiratory distress syndrome in pediatric intensive care unit at Queen Sirikit National Institute of Child Health [thesis]. Bangkok: Queen Sirikit National Institute of Child Health; 2009.

Wang P, Dong L, Zhang L, Xia LJ. Etiology and epidemic characteristics of hospital acquired pneumonia in children. *Zhonghua Er Ke Za Zhi* 2010; 48: 465-8. [in Chinese]

Waqar AB, Khan S, Idrees M. Novelity in GB virus C/hepatitis G virus and its controversy. *J Ayub Med Coll Abbottabad* 2002; 14: 31-5.

Wilson MR, Naccache SN, Samayoa E, *et al*. Actionable diagnosis of neuroleptospirosis by next-generation sequencing. *N Engl J Med* 2014; 370: 2408-17.

Wiwanitkit V. New emerging blood-borne hepatitis viral pathogens and the feasibility of passing thorough the placenta: an appraisal. *Clin Exp Obstet Gynecol* 2006; 33:213-4.

Woo PC, Lau SK, Chu CM, *et al*. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. *J Virol* 2005; 79: 884-95.

Woo PC, Lau SK, Yip CC, Huang Y, Yuen KY. More and more coronaviruses: human coronavirus HKU1. *Viruses* 2009; 1: 57-71.

Yee-Guardino S, Danziger-Isakov L, Knouse M, Bingaman W, Sabella C, Goldfarb J. Nosocomially acquired *Pseudomonas stutzeri* brain abscess in a child: case report and review. *Infect Control Hosp Epidemiol* 2006; 27: 630-2.

Yu D, Yin Z, Li B, *et al*. Gene flow, recombination, and positive selection in *Stenotrophomonas maltophilia*: mechanisms underlying the diversity of the widespread opportunistic pathogen. *Genome* 2016; 59: 1063-75.

Yuan SM. Mycobacterial endocarditis: a comprehensive review. *Rev Bras Cir Cardiovasc* 2015; 30: 93-103.