การใช้ multiplex PCR ในการตรวจหาเชื้อก่อโรครุนแรงคุกคามต่อชีวิตในเด็กที่มีสุขภาพแข็งแรงดีมาก่อน

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Abstract: Multiplex PCR for Pathogen Identification among Otherwise Healthy Children Presenting with AcuteLife-threatening Infection

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Background: Diagnostic yield of conventional, culture-based method of pathogen detection among sepsis and severe pneumonia is generally disappointedly low. Objectives: To determine the etiological organisms causing community-acquired life-threatening infections (CA-LTI) among previously healthy children using multiplex polymerase chain reaction (mPCR). Methods: A prospective descriptive survey was conducted among all children aged 1 month -15 years diagnosed with community-acquired sepsis with septic shock and pneumonia with respiratory failure. Serum and/or tracheal aspirates for pathogen identification using mPCR targeting 33 common pathogens were obtained from all subjects. Results: A total of 79 cases were enrolled: n = 17 for sepsis (21.5%) and n = 62 for pneumonia (78.5%). Nine cases (11.4%) had one pathogen identified in serum sample using mPCR: n = 4 (23.5%) for sepsis and n=5 (8%) for pneumonia cases. Among sepsis cases, the pathogens identified in serum via mPCR were rhinovirus and bocavirus, (n=2 each). One pathogen identified by conventional blood culture was Entercoccus faecalis. Among pneumonia cases, 8% (n=5) had at least one potential pathogen identified in serum by mPCR with bocavirus and H. influenzae being the most common (n= 2 each). Similarly, H. influenzae were the most commonly identified by mPCR in tracheal aspirates (n=10). The fatality rates were 5.9% among sepsis and 1.6% among pneumonia cases. Conclusions: Viruses are among the important causes of CA-LTI in previously healthy children. Using mPCR enables 9-fold increase in the proportion of pathogen identification in serum in children with septic shock and severe pneumonia.

Keywords: Sepsis, Septic shock, Pneumonia, Respiratory failure, Multiplex polymerase chain reaction

บทคัดย่อ

ภูมิหลัง: การตรวจเชื้อแบบดั้งเดิมเพื่อหาสาเหตุการติดเชื้อในกระแสเลือด หรือกลุ่มอาการ sepsis และภาวะปอดอักเสบรุนแรงมีความไวค่อนข้างต่ำ **วัตถุประสงค์:** ศึกษาวิธีการตรวจในรูปแบบ multiplex polymerase chain reaction (mPCR) เพื่อตรวจหาเชื้อก่อโรคในผู้ป่วยเด็กที่ไม่มีโรคประจำตัวและมีสุขภาพแข็งแรงดีมาก่อน แต่มาด้วยอาการเจ็บป่วยเฉียบพลัน ติดเชื้อรุนแรงคุกคามต่อชีวิต **วิธีการ:** สำรวจเด็กอายุ 1 เดือนถึง 15 ปีที่ได้รับการวินิจฉัยว่ามีการติดเชื้อรุนแรงและคุกคามต่อชีวิตด้วยกลุ่มอาการ sepsis ร่วมกับภาวะช็อกและหรือมีภาวะปอดอักเสบรุนแรง โดยนำเลือดและน้ำจากท่อหลอดลมคอส่งตรวจโดยวิธี mPCR ซึ่งครอบคลุมเชื้อ 33 ชนิดที่พบบ่อย **ผล:** เด็กเข้าร่วมทั้งหมด 79 ราย แบ่งเป็นกลุ่มอาการ septic shock 17 ราย (ร้อยละ 21) ปอดอักเสบรุนแรง 62 ราย (ร้อยละ 78.5) พบว่า 9 ราย (ร้อยละ 11.4) ตรวจพบเชื้อก่อโรคในเลือด แบ่งเป็นกลุ่มอาการ sepsis 4 ราย (ร้อยละ 23.5) ปอดอักเสบ 5 ราย (ร้อยละ 8) กลุ่มอาการ septic shock เชื้อที่ตรวจพบในเลือด ได้แก่ rhinovirus และ bocavirus (อย่างละ 2 ราย) มี 1 ราย ตรวจพบเชื้อ Entercoccus faecalis โดยวิธีเพาะเชื้อแบบปกติ กลุ่มอาการปอดอักเสบ 5 ราย (ร้อยละ 8) พบเชื้ออย่างน้อย 1 ชนิดโดยวิธี mPCR โดย bocavirus และ H. influenzae เป็นเชื้อที่พบบ่อยที่สุด (อย่างละ 2 ราย) โดยวิธี mPCR จากน้ำในท่อหลอดลมคอ (10 ราย) อัตราการเสียชีวิต พบร้อยละ 5.9 ในกลุ่มอาการ septic shock ร้อยละ 1.6 ในปอดอักเสบ **สรุป:** เชื้อไวรัสเป็นสาเหตุสำคัญที่ตรวจพบในเด็กที่มีสุขภาพแข็งแรงดีมาก่อนแต่มาด้วยอาการติดเชื้อรุนแรงคุกคามต่อชีวิต การใช้ mPCR ในเด็กที่มาด้วย กลุ่มอาการ septic shock และหรือปอดอักเสบรุนแรง ทำให้ตรวจพบเชื้อก่อโรคได้เพิ่มขึ้นในเลือดเมื่อเทียบกับการตรวจวิธีปกติประมาณ 9 เท่า

คำสำคัญ: การติดเชื้อในกระแสเลือด ภาวะช็อคจากการติดเชื้อในกระแสเลือด ปอดอักเสบ หายใจล้มเหลว การตรวจหาเชื้อก่อโรครุนแรงคุกคามต่อชีวิต

Introduction

Sepsis is a serious, life-threatening condition and a leading cause of death in hospitalized patients worldwide despite advances in modern medicine, including vaccines, antibiotics, and access to care.[1-4] Sepsis occurs when the body's response to infection damages its own organs and tissues which may resulting in circulatory/organ failure, and death, particularly without early recognition and timely intervention. Childhood pneumonia is also an important cause pediatric sepsis and major contributor for the mortality in children under 5 years of age.[5] Although countries with large population density and limited resources generally bear the major burden of childhood pneumonia and sepsis, little data are available regarding causative pathogens. Therefore, the current management strategies for these two life threatening condition remain less than optimal due mainly to the lack of adequate pathogen identification tools to determine etiology and predict their outcomes.

Culture has been regarded as the "gold standard" for the identification of pathogens causing these 2 serious and life threatening conditions. Nevertheless, culture is labor-intensive, and time-consuming. In parallel, the low detection sensitivity of traditional bacterial culture had result in the lack of clear etiologic diagnosis in these two serious infections. Further, the conventional laboratory tests for pneumonia pathogens had low diagnostic value that current clinical practice guidelines do not recommend testing for any but the most severely affected individuals. Molecular diagnostics may, therefore, offer an important opportunity to narrow this knowledge gap.

Due to the high number of etiological agents potentially implicated in respiratory infections, the use of conventional monoplex polymerase chain reaction (PCR) for this condition become expensive, labor-intensive, and require large amounts of samples.[6,7] Therefore, multiplex PCR is thus considered alternatives to obtain faster results and higher sensitivity and specificity.[8,9] A number of commercial assays have been utilized for diagnostic purpose that allow simultaneously detection of high number (12-33) of different pathogens.[10] In this study, we aimed to determine etiological agents causing community-acquired life-threatening infections (CA-LTI) in previously healthy children using a multiplex polymerase chain reaction (mPCR) targeting 33 common pathogens in addition to conventional laboratory investigation. Information obtained from this study may be used as a guidance for future empirical treatment for children with similar conditions. Further, we will also be able to evaluate the additional benefit of mPCR in identifying potential pathogens causing CA-LTI.

Materials and Methods

A prospective descriptive survey was conducted among previously healthy children aged between 1 month to 15 years hospitalized at Queen Sirikit National Institute of Child Health (QSNICH) with community onset of sepsis with septic shock and/or pneumonia with respiratory failure during the period between September 2014-October 2015. Sepsis was defined as cases with signs of systemic inflammatory response plus a confirmed or presumed infection according to the criteria proposed by Goldstein and colleagues.[11] Septic shock was defined clinically as sepsis cases with evidence of hypotension that was not resolved despite adequate fluid resuscitation.[11] Pneumonia was diagnosed clinically by the presence of respiratory distress and abnormal lung auscultation and pulmonary infiltrates on chest x-ray presumably caused by infectious agent. All eligible subjects were approached and those provided written informed consent by caregivers were enrolled consecutive during the study period. Clinical information and laboratory findings were abstracted from medical records. Additional serum and/or tracheal aspirate specimen (for those intubated) were sent for pathogen identification using a commercially available multiplex polymerase chain reaction (mPCR) at the Armed Force Research Institute of Medical Science, Bangkok, Thailand. The assay used in this study was FTD Respiratory pathogens 33 (Fast-track Diagnostics Luxembourg) which was based on multiplex one-step reverse transcription polymerase chain reactions with probes for detecting 33 common respiratory pathogens (21 viruses, 11 bacteria, and 1 fungus). It was used with the Bio-Rad CFX96 thermocycler and the SuperScript III Platinum One-Step Quantitative RT-PCR System without ROX (Invitrogen). The list of common pathogens included in this assays is as follows: influenza A, B and C; parainfluenza viruses 1, 2, 3 and 4; coronaviruses NL63, 229E, OC43 and HKU1; human metapneumoviruses A and B; rhinovirus; respiratory syncytial viruses (RSV) A and B; adenovirus; enterovirus; parechovirus; bocavirus; cytomegalovirus; *Pneumocystis jirovecii (PCP); Mycoplasma pneumoniae; Chlamydia pneumoniae; Streptococcus pneumoniae; Haemophilus influenzae type B; Staphylococcus aureus; Moraxella catarrhalis; Bordetella pertussis; Klebsiella pneumoniae; Legionella spp.; Salmonella spp.;* and *Haemophilus influenzae.* The testing method was performed according to the manufacturer's instructions. This assay has been reported to provide high diagnostic performance with good Youden's index, high positive predictive and specificity value.[10]

In this study, organisms identified by conventional blood culture and mPCR in serum (normally sterile site) were regarded as causative pathogen. Organisms identified in tracheal aspirates were considered as potential ones. The data were descriptively analysed and reported. We conducted a post hoc analysis.

The Research Ethical Committee of Queen Sirikit National Institute of Child Health approved this study.

Results

Table 1 Distribution of potential pathogens identified by multiplex PCR in sepsis cases (n=17)

Pathogens in sepsis cases	n (%)
Types of organism identified in serum	
Bocavirus	2 (11.8)
Rhinovirus	2 (11.8)

Table 1 Distribution of potential pathogens identified by multiplex PCR in sepsis cases (n=17) (continue)

Pathogens in sepsis cases	n (%)
Types of organism identified in tracheal aspirate	
Haemophilus influenza	4 (23.5)
Rhinovirus	3 (17.6)
Parainfluenza virus	2 (11.8)
Moraxella cattarhalis	2 (11.8)
Enterovirus	1 (5.9)
Adenovirus	1 (5.9)
Cytomegalovirus	1 (5.9)
Streptococcus pneumoniae	1 (5.9)

*PCR: polymerase chain reaction

A total of 79 cases were enrolled: n= 17 for sepsis (21.5%) and n= 62 for pneumonia (78.5%). Nine cases (11.4%) had one pathogen identified in serum sample using mPCR: n=4 (23.5%) for sepsis and n=5 (8%) for pneumonia cases. Among 17 sepsis cases, the pathogens identified in serum via mPCR were rhinovirus and bocavirus, (n=2 each) (Table 1). One pathogen identified by conventional blood culture, but not mPCR, was Entercoccus faecalis which was not a part of multiplex panel. One sepsis case (5.8%) had rhinovirus concurrent identified in both blood and tracheal aspirates. In total, using both mPCR and conventional blood culture, causative pathogen were identified in 29.4 % (5/17) of sepsis cases where viral pathogens were major organisms associated with this condition (23.5%).

Table 2 Distribution of potential pathogens identified by multiplex PCR in pneumonia cases with respiratory failure (n=62)

Pathogens in sepsis cases	n (%)
Types of organism identified in serum	
Bocavirus	2 (3.2)
Salmonella sp, Haemophilus influenzae	1 (1.6)
Haemophilus influenzae (nontypable)	1 (1.6)
Legionella sp.	1 (1.6)
Types of organism identified in tracheal aspirates	
Haemophilus influenzae	10 (16.1)
Respiratory syncytial virus	9 (14.5)
Rhinovirus	8 (12.9)
Streptococcus pneumoniae	7 (11.3)
Moraxella catarrhalis	6 (9.7)
Parainfluenzae	3 (4.8)

Table 2 Distribution of potential pathogens identified by multiplex PCR in pneumonia cases with respiratory failure (n=62) (continue)

Pathogens in sepsis cases	n (%)
Pneumocystis jirovecii	2 (3.2)
Klebsiella pnemoniae	2 (3.2)
Staphylococcus aureus	2 (3.2)
Cytomegalovirus	2 (3.2)
Metapneumovirus	(3.2) 2
Adenovirus	1 (1.6)
Enterovirus	1 (1.6)
Influenza virus	1 (1.6)

* Some cases had more than one organism identified, CAP: community-acquired pneumonia, PCR: polymerase chain reaction)

In 62 pneumonia cases, 8% (n=5) and 41.9% (n= 26) had at least one potential pathogen identified in serum and tracheal aspirates, respectively. While bocavirus was the most common organism pathogens identified in serum, H. influenzae (nontypable) was the most commonly identified organism in tracheal aspirates (16.1%), followed by RSV (14.5%) and rhinovirus (12.9%), respectively (Table 2).

Table 3 Distribution of pathogens identified from tracheal aspirates by multiplex PCR

Tracheal aspirates	Sepsis with shock (n=17) n (%)	CAP with respiratory failure (n=62) n (%)
At least one potential pathogen identified	9 (47.1)	26 (41.9)
Single virus	3 (17.6)	11 (17.7)
Multiple viruses	0 (0)	2 (3.2)
Single bacteria	1 (5.9)	1 (1.6)
Multiple bacteria	0 (0)	1 (1.6)
Mixed viruses and bacteria	4 (23.5)	11 (17.7)

*CAP: community-acquired pneumonia; PCR: polymerase chain reaction

Table 3 demonstrates the patterns of pathogens identified from tracheal aspirates where mixed virus and bacteria were the most common pattern detected followed by single virus for both groups.

The overall fatality rate were 2.5% (n=2) i.e., 5.9% (1/17) among sepsis and 1.6% (1/62) among pneumonia cases. The potential pathogens identified in 2 fatal cases were from tracheal aspirates including enterovirus in one sepsis case and adenovirus plus Pneumocystis jirovecii in one pneumonia case. We did not identified any potential pathogens in serum, however, by either conventional culture or mPCR in both fatal cases.

To explore some predictors for disease outcomes, we conducted a post hoc analysis using using the composite outcome of mortality and/or prolonged hospitalization (more than 2-week duration) as a surrogate for unfavourable outcome. The presence of viral and/or bacterial pathogens in serum sample by either mPCR or conventional culture did not appear to be associated with poor outcome in both sepsis (RR 0.81, 95% CI=0.28, 2.36) and pneumonia groups (RR = 0.63, 95% CI=0.11, 3.81) by univariate analyses. Due to the limited number of cases, we were unable to identify independent prognostic factors in this sample.

Discussion

To reduce mortality of these two major life threatening infections, local epidemiological data on causative pathogen are essential to guide appropriate empirical treatment. Nevertheless, data on etiological agents for these conditions are limited especially in resource-poor settings where sensitive diagnostic methods are generally unavailable.

In general, the conventional blood culture are considered a gold standard for the recovery of bacterial pathogens. Nevertheless, the process is generally hampered by a low degree of bacteraemia, certain characteristics of the particular pathogens e.g., nonbacterial agents, presence of intracellular, fastidious, or slow-growing types of organisms, prior antibiotic exposure, limited volume of blood being obtained, timing and method of collection.[12,13] This study was, therefore, set out to identify the potential pathogens of CA-LTI, not typically captured by conventional culture system, using molecular diagnoistic method. In addition, we also would like to determine the additional benefit of mPCR in providing an important insight on the etiology of this condition. Our findings indicated that only one case (6%) had positive blood culture which was an unusual cause of sepsis i.e., Enterococcus fecalis. Further, despite the highly sensitive molecular technology and severity of the sepsis symptoms, the yield of this methods remained rather low. In other words, approximately one fourth of sepsis cases had potential pathogens identified in blood samples by mPCR, all of which were viral organisms. Consistent with existing literature that despite the potential benefit of mPCR for pathogen detection in sepsis case, the identification rate remained rather low.[14]

In this study, human bocavirus was the leading viral agent identified in blood samples from both sepsis and pneumonia cases. According to the current review published in 2016.[15] Indicated that HBoV mainly affects infants aged 6-24 months with respiratory illness.[16] However, according to Koch’s modified postulates, the virus cannot yet be established as a causative pathogen for human disease due to the lack of animal models and specific cell line for in vitro viral replication[17-19] In addition to respiratory samples, bocavirus has been reported to be detected in normally sterile site such as blood and cerebrospinal fluid. As well as other biological and environmental samples[12]

Various reports demonstrated the role of viral pathogens in causing sepsis-like illness with septic shock in various population settings such as late onset-neonatal sepsis.[20,21] In one study, respiratory virus was detected in 6.8% of sepsis cases.[16] Further, enterovirus were detected in 24% of blood samples of 139 neonates admitted to hospital with sepsis-like illness with negative bacterial cultures.[21] In addition, the outbreak of human parechovirus type 3 (HPeV3) epidemic in Niigata, Japan in 2014 has showed that 43 cases of HPcV3 infection (with positive blood and/or cerebrospinal fluid PCR) were diagnosed as having sepsis (79%), sepsis-like syndrome (19%), or encephalitis with septic shock (2%).[22] Another report on Chikungunya outbreak in 2014 indicated that 5.6% of hospitalized cases developed severe sepsis or septic shock and with 2.6% fatality rate.

Despite the higher sensitivity of PCR compared to blood culture, limited data are available regarding the significance of positive PCR without concurrent positive blood culture results. Bloos and colleagues evaluated the association of PCR status with disease severity in an attempt to validate the significance of the positive nucleic amplification without positive blood culture. In this study, there was a moderate concordance between blood culture and PCR. They found that those with positive

PCR had higher organ dysfunction scores and a trend toward higher mortality rates (39.1% vs. 25.3% among those with and without positive PCR, respectively). These findings lend support to the notion that positive PCR was clinically meaningful among sepsis cases.[23]

Among pneumonia cases, our findings were rather in concordance with a recent meta-analysis on the role of viral infection in childhood CAP i.e., rhinovirus, RSV and bocavirus were the three most common viral pathogens.[24] In this review, the mixed infection was identified in 29.3%. The detection rate of respiratory viruses varied with age with 76.1% in patients aged 1 year or younger, 63.1% in patients aged 2-5 years and 27.9% of patients aged ≥ 6 years. More than half of viral infections were probably concurrent with bacterial infections.[24]

Similarly, another related study indicated that the use of mPCR including 23 pathogenic respiratory agents in detecting respiratory virus in outpatient with acute respiratory infections provided 54.3% positive results.[17] Additionally, the most prevalent viral pathogens were influenza, human rhinovirus and RSV. The use of mPCR increased the viral detection by 33.9% and uncovered a larger number of respiratory viruses implicated in ARI cases e.g., human coronavirus (HCoV) NL63 and HCoV HKU1.[25]

Further findings from a recent report from Thailand among children with severe CAP also lend further support to our findings.[26] They found that the most commonly isolated viral pathogens from nasopharyngeal secretion were RSV (n = 22; 45.8%), rhinovirus (n = 11; 22.9%) and adenovirus (n = 9; 18.7%)

Of note, although respiratory viruses were the common organisms identified in our clinical samples, influenza, the only viral pathogen with specific treatment, was identified in tracheal aspirates in only 2 cases (2.5%) suggesting potential minor impact of routine use of oseltamivir in this population.

Limitations: The findings of this study should be interpreted with caution due to the potential limitation as follows. First, asymptomatic carriage or persistent infection of common respiratory viruses causing CAP could be found for several months among immunocompromised individuals or patient with underlying respiratory diseases.[27,28,29] Second, the viruses could be detected for several days prior to the symptom onset as well as after clinical recovery. In addition, children appear to have higher level and longer duration of viral shedding compared to adults. It is common belief that asymptomatic carrier of these virus do not occur in healthy individuals outside the abovementioned period. Nevertheless, subclinical or mildly symptomatic infections are common which can be easily unrecognized thus leading to diagnostic confusion. In parallel, there is growing evidence that bacteria or its component can be detected in blood of asymptomatic individuals. Several studies have reported the detection of bacterial DNA in blood from healthy subjects and explained such detection as being secondary to carriage, contamination, or simply a reservoir of low pathogenic bacteria, commonly belonged to normal flora.[30,31]

Kumar and colleagues attempted to determined the prevalence of carriage of potential respiratory pathogens in respiratory, skin, and blood samples from 243 asymptomatic children and adults using molecular assay of 11 common viral and bacterial agents of CAP and sepsis. The assay included influenza virus A, influenza virus B, RSV A, RSV B, *Mycoplasma pneumoniae, Chlamydophila pneumoniae, Legionella pneumophila, Legionella micdadei, Bordetella pertussis,* Staphylococcus aureus, and Streptococcus pneumoniae. The results indicated that 21.7% of nasopharyngeal specimens, 11% of skin specimens, and 2% of serum specimens from asymptomatic subjects tested positive for one or more pathogens, with S. pneumoniae and S. aureus were responsible for 89% of the positive results. This finding indicated that asymptomatic carriage renders the use of molecular assays problematic for the detection of *S. pneumoniae or S. aureus* in upper respiratory samples but there were virtually no carriage of the other nine viral and bacterial pathogens. As a result, the identification of these pathogens was unlikely to cause significant diagnostic problem. Another important limitation of highly sensitive amplification tests is DNA contamination of PCR reagents and other laboratory materials.[18] This poses an especially challenging concern when trying to distinguish a small degree of bacterial DNA in clinical samples and

contamination from specimen processing and testing procedures.

Of important note, with advance molecular technologies, viruses were increasingly isolated in children and adults with pneumonia.[32] Nevertheless, the lack of standardization and substantial variation of molecular testing have been criticized for its validity and reproducibility. Therefore, it remained uncertain to establish its clear etiologic role in this condition.[33] For example, certain potential pathogens commonly found in airway and/or sera of asymptomatic controls rendering the difficulties in interpretation of results. In addition, the role of viral-bacterial synergistic interactions on the pathogenesis of the disease make it difficult to reach the final conclusion regarding the role of these organisms detected in respiratory airway. Therefore it may be important to apply the molecular technology concurrently in samples from both blood and respiratory airway to establish the invasiveness of potential pathogens as we have done in this study.

Despite potential weaknesses, this study provides important insight into the etiology and guidance to optimal intervention. Physicians tend to focus mainly for the role of bacterial pathogens in serious infection such as severe pneumonia and sepsis especially in young children. Therefore, broad-spectrum antibiotics are likely to be prescribed and rapidly escalated if there is no significant improvement. Physicians are rather reluctant to discontinue or step down despite the negative culture due to the low sensitivity of conventional culture. The increasing use of broad-spectrum antibiotics has led to the threat of antimicrobial resistance. This finding suggests that viral pathogens play important roles of sepsis and thus antibiotic may not need to be used in the extended period once the patient become stabilized and no evidence of invasive bacterial infection has been documented.

Conclusions

Pathogen identification remains a daunting challenge in the diagnosis of community-acquired life threatening infection in children. Major potential pathogens identified in acute life threatening infection in this study were viral agents especially bocavirus. Therefore, fluid resuscitation and supportive treatment is particularly essential as no specific antiviral is currently available. mPCR helps uncovered unusual pathogens as well as common bacterial causes of CA-LTI undetected by conventional bacterial culture. Although mPCR has 9-fold increased the rate of potential pathogen identification, the rate of pathogen identified in serum remain low. Further studies with larger sample are needed to determine the proper antibiotic options for these conditions and the performance of molecular diagnosis compared to conventional cultures.

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