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Multiplex real-time PCR assay for detection of respiratory pathogens among pneumonia affected children

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Abstract: Molecular diagnostic methods offer a rapid and more sensitive strategy with high selectivity compared to the traditional immunoassays and culture techniques. Present study attempted to employ the molecular approach for the diagnosis of respiratory viral- and bacterial infections among the pneumonia infected children in Dhaka Metropolis. Samples were aseptically collected, processed and analyzed by using Multiplex Real Time PCR technique to detect respiratory pathogens within a time frame of February 2013-June 2013. Influenza viruses, human meta-pneumo viruses, respiratory syncytial viruses and other potential infective viruses were diagnosed. Among the infective bacterial population, presence of Mycoplasma pneumoniae, Chlamydia pneumoniae, Streptococcus pneumoniae, Staphylococcus aureus, Klebsiella pneumoniae, Legionella species, Salmonella species were detected. Among the nasopharyngeal and orpharyngeal (NPOP) samples, Influenza B and Streptococcus pneumoniae were found to be dominating (34.8% and 30.4%, respectively) whereas the induced sputum (IS) samples were 100% positive for S. pneumoniae followed by around 50% prevalence with equine arteritis virus, para influenza virus and the respiratory syncytial viruses A and B. Considering the spatial identification of an array of microorganisms, results of the current study sufficiently may endorse such molecular method of diagnosis for the betterment of public health.

Keywords: Respiratory Pathogens, Pneumonia, Molecular Diagnostic Methods, Public Health

1. Introduction

Acute respiratory tract infections have long been known to be the leading cause of hospitalization of infants and young children in developing countries with a subsequent impact on children death [1-3]. Pneumonia, an infection of the lungs, is a serious illness with significant morbidity and mortality rates. A wide range of microorganisms may trigger pneumonia in children as well as in adults; however, the exact etiology in 20-50% cases has not yet been established [4,5]. Around 20% Bangladeshi children (<2 years) with respiratory infection unfortunately can not avail any health care services [6]. Furthermore, malnutrition, overcrowding and lack of access to health care may contribute to the increased mortality [7,8]. Therefore, easy, accurate and rapid molecular diagnosis of a broad range of respiratory pathogens are in demand alternative to the conventional culture methods which often leads to false negative results.

Interestingly, the prevalence of pneumonia is concentrated in India, China, Pakistan, Bangladesh, Indonesia and Nigeria where 44% of the world's children aged less than 5 years live [9-14]. Along with other infectious diseases, pneumonia stands as the trigger of death among children in low income countries [11,15-20]. However, limited information is available on the severity of risk factors associated with pneumonia among young children in this region. Besides, lack of sufficient knowledge on the molecular diagnostic strategy may interrupt the precise method development.

For the past two decades, molecular diagnostic techniques for the detection of respiratory pathogens over the conventional culture techniques have developed globally, providing rapid results with an increased sensitivity [11, 21-23]. Among them, the multiplex real-time PCR has been shown to be more sensitive and specific, yielding results within 6 hours and enabling the direct detection of viruses which is impossible only be employing the culture methods [24-26].

Along these lines, present study attempted to estimate the incidence of pneumonia and detection of causative

agents of this disease in the developing countries like Bangladesh.

2. Materials and Methods

2.1. Sampling

Respiratory samples including nasopharyngeal (NP), orpharyngeal (OP) and induced sputum (IS) were collected, processed and analyzed during February 2013-June 2013. Two nasopharyngeal (NP) specimens were collected from each of the pneumonia infected children. One NP flocked swab was collected placed into viral transport medium, and a second NP Dacron or rayon swab was placed in skim milk tryptone - glucose –glycerin (STGG) medium. Orpharyngeal (OP) Dacron or rayon swab was collected and placed into the same vial as the NP flocked swab. The OP swab was taken by touching the swab to the posterior oropharynx and rubbing for 1-3 seconds. One ml of induced sputum was collected from all patients within 24 hours except those with contraindications, which include the following: hypoxia (<92% on supplemental oxygen), inability to protect airways, and severe bronchospasm at admission in hospital. Metered Dose Inhalers (MDIs) was used for bronchodilation. Light chest percussion was employed in children <2 years to improve mobilization of sputum. In children ≥2 years, mobilized sputum was collected by induction with nebulization of normal saline. The quality of the specimen was assessed by using the "Bartlett's Score". All samples were stored at −20 C until extraction.

2.2. Preliminary Extraction of Nucleic Acid with EasyMag (BioMérieux) and Selection of Primer/Probe

One negative control (NC) and one internal control (IC) was taken from the refrigerator and kept in the normal temperature for some minutes. All the organisms described in table 1 were used as positive and negative controls. For example, PC fluAB_RHPPmix =Master mix and positive control, NC fluAB_RHPPmix = Master mix and negative control. All the PC & NC were free from extracted samples. Positive controls were not extracted. Equine arteritis virus was used as an internal control (IC), which was introduced into the lysis buffer at the extraction stage of each sample and the negative control. Before use, reagents were thawed and homogeneously mixed. Patient samples and the NC were extracted by an automated nucleic acid extractor (NucliSENS™ EasyMAG, USA). The starting volume for the extraction was 400 µl and an elution volume was 110 µl. Four µl of the internal controls was added to the lysis buffer of each extraction.

To perform real time PCR, different primer/ probe mix were used for pathogen detection (Table 1). In the present study, Influenza A, B and C viruses, parainfluenza viruses 1, 2, 3 and 4, coronaviruses, human meta-pneumoviruses, rhinoviruses, respiratory syncytial viruses, adenoviruses, enteroviruses, parechoviruses, bocaviruses, cytomegaloviruses, Pneumocystis jirovecii, Mycoplasma pneumoniae), Chlamydia pneumonia, Streptococcus pneumoniae, Haemophilus influenza, Staphylococcus aureus, Klebsiella pneumonia, Legionella spp., Salmonella spp. and Haemophilus influenzae were detected.

2.2.1. Preparation of PCR with AgPath- ID™One-Step RT-PCR Kit (Ambion®)

FluAB_RH PP mix, Para.EAV PP mix, Cor PP mix, BoMpPf1 PP mix, RsEPAcmv PP mix, Bac PP mix, KLePSa PP mix, MoBoCH PP mix, PC1_Resp33_ pos_ plmdpool, PC2_ Resp33_ pos_ plmd pool and the "2x RT PCR buffer" of AgPath-ID™ One-Step RT-PCR Kit were thawed for the PCR reaction. For one reaction 12.5µl of the "2x RT-PCR buffer" of AgPath-ID™ One-Step RT-PCR Kit was homogeneously mixed with 1.5µl PP mixes. One µl of the "25x RT PCR enzyme mix" of AgPath-ID™One-Step RT-PCR Kit was added to the 1.5µl PP mixes with the "2x RT-PCR buffer".

Table 1. *Label of information of analyzed primer/probe*

Label	Contents
FluAB_RH PP	Primer/probe mix for Flu A, B & Rhino
Para.EAV PP	Primer/probe mix for Para 2,3, 4 & IC-EAV
Cor PP	Primer/probe mix for Cor 43,63, 229 & HKU1
BoMpPf1 PP	Primer/probe mix for HboV, Mpneu, Para1 & HMPVA/B
RsEPAcmv PP	Primer/probe mix for RSVA/B, CMV, AV & EV/PV
Bac PP	Primer/probe mix for Saur, Spneu, Cpneu & HIB
KLePSa PP	Primer/probe mix for Kpneu, Legio, PCP &Salm
MoBoCH PP	Primer/probe mix for Morax, Bord, FluC & Haeinf
Resp PC1 Plasmids	For use with the yellow PPmixes 1-5 (FluA/B, Rhino, Para 1/2/3/4, Cor43/ 63/ 229/ HKU, HboV, Mpneu, HMPVA/B, RSVA/B, PV/EV, AV and CMV).
Resp PC2 Plasmids	For use with the red PPmixes 6-8 (Saur, Spneu, Cpneu, HIB, Kpneu, Legio, PCP, Salm, Morax, Bord, FluC and Haeinf).
Resp NC	Negative control
Resp IC	Internal control

Key: PP- primer and probe, IC- internal control, PC- positive control, NC- negative control, Flu- Influenza, Para- Para influenza virus, Cor- Corona virus, HMPV- Human meta pneumo virus, Rhino- Rhinovirus, RSV- Respiratory syncytial virus, AV- Adenovirus, EV- Enterovirus, PV- Parechovirus, HMPVA- Human meta-pneumoviruses, HboV- Bocavirus, CMV- Cytomegalovirus, PCP- Pneumocystis jirovecii, Mpneu- Mycoplasma pneumoniae, Cpneu- Chlamydia pneumoniae, Spneu- Streptococcus pneumoniae, HIB- Haemophilus influenzae type B, Saur- Staphylococcus aureus, Morax- Moraxella catarrhalis:, Bord- Bordetella pertussis, Kpneu- Klebsiella pneumoniae,

Legio- Legionella spp., Salm- Salmonella spp., Haeinf- Haemophilus influenzae.

2.3. Preparation of a 96 Well Plate for ABI 7500 (Applied Biosystems®)

A 96 well plate (ABI 7500) was taken. For 1 reaction, 15 µl of the previously mentioned different PP mixes was mixed with the PCR buffer and the PCR enzyme mix in each of the wells. Ten µl of the extracted samples, the extracted negative control and the positive control (which was not extracted) was added to each mix (Each run was included with a negative and a positive control). The plate was closed with the ABI optical adhesive film, and then centrifuged.

2.4. Programming of the Thermo Cycler

The presence of specific viral and bacterial sequences in the reaction was detected by an increase in the fluorescence (ROX dye) observed from the relevant dual-labeled probe by the real time thermo cycler [27]. Eight different types of primer/probe mix were used to detect specific respiratory pathogens (Table 2).

Table 2. *Settings of the detectors*

PP Mix	Pathogen	Reporter	Fluorescent Dye	Detection wavelength (mm)
FluAB_RH PP mix	FluA	FAM	Green	520
	Rhino	VIC	Yellow	550
	FluB	ROX	Orange	610
Para.EAV PP mix	Para 3	FAM	Green	520
	Para 2	VIC	Yellow	550
	Para 4	ROX	Orange	610
	IC (EAV)	Cy5	Red	670
Cor PP mix	Cor 229	FAM	Green	520
	Cor 63	YAK	Yellow	550
	HKU1	ROX	Orange	610
	Cor 43	Cy5	Red	670
BoMpPf1 PPmix	Para 1	FAM	Green	520
	HMPV A/B	VIC	Yellow	550
	HBoV	ROX	Orange	610
	Mpneu	Cy5	Red	670
RsEPAcmv PP mix	RSVA/B	FAM	Green	520
	CMV	VIC	Yellow	550
	EV/PV	ROX	Orange	610
	AV	ATTO-647N*	Red	670
Bac PPmix	S.aur	FAM	Green	520
	C.pneu	VIC	Yellow	550
	HiB	ROX	Orange	610
	S.pneu	Cy5	Red	670
KLePsa PPmix	PCP	FAM	Green	520
	Legio	VIC	Yellow	550
	K.pneu	ROX	Orange	610
	Salm	Cy5	Red	670
MoBoCH PPmix	Morax	FAM	Green	520
	FluC	VIC	Yellow	550
	Bord	ROX	Orange	610
	Haeinf	ATTO-647N	Red	670

3. Results and Discussion

3.1. Prevalence of Respiratory Pathogens

In the present study, out of 35 cases, in 23 nasopharyngeal and orpharyngealswab (NPOP) samples, 28 pathogens were detected, while in 12 induced sputum (IS) samples, a total of 20 virus and bacteria were identified. In NPOP samples, Influenza B and S. pneumoniae possessed a relatively high frequency (34.8% and 30.4%, respectively), whereas parainfluenza viruses, human metapneumoviruses A and B, respiratory syncytial viruses A and B, S. aureus and Influenza C were found with a low frequency (Table 3). On the other hand, among the 12 IS samples, S. pneumoniae, Equine arteritis virus, Para influenza virus 1 and respiratory syncytial viruses A and B were in high frequency while influenza A , parainfluenza viruses, human metapneumoviruses A and B were found to be in a relatively low frequency (Table 3).

Table 3. *Frequency of pathogens*

Pathogens	No. of Positive Samples		%Frequency	
	NPOP	IS	NPOP	IS
FluA	5	1	21.7%	8.3%
Rhino	3	0	13.04%	0%
FluB	8	6	34.8.0%	50.0%
Para 3	1	2	4.4%	16.7%
Para 2	2	1	8.7%	8.3%
Para 4	1	1	4.4%	8.3%
IC(EAV)	3	7	13.04%	58.3%
Cor 229	3	0	13.04%	0%
Cor 63	3	0	13.04%	0%
HKU1	6	0	26.09%	0%
Cor 43	6	0	26.09%	0%
Para 1	5	7	21.7%	58.3%
HMPVA/B	1	1	4.4%	8.3%
HBoV	6	5	26.09%	41.7%
Mpneu	3	3	13.4%	25.0%
RSVA/B	1	7	4.4%	58.3%
CMV	1	3	4.4%	25.0%
EV/PV	0	2	0%	16.7%
AV	4	0	17.3%	0%
Saur	1	0	4.4%	0%
Cpneu	2	6	8.7%	50.0%
HiB	5	0	21.7%	0%
Spneu	7	12	30.4%	100%
PCP	4	5	17.3%	41.7%
Legio	3	2	13.04%	16.7%
Kpneu	3	3	13.04%	25.0%
Salm	3	3	13.04%	25.0%
Morax	0	0	0%	0%
FluC	1	0	4.4%	0%
Bord	0	0	0%	0%
Haeinf	3	2	13.04%	16.7%

However, a limitation of the study may lies under the fact that several variables might hinder the adoption of such diagnostic assays based on the pathogen's DNA, rather than on its phenotype including the probable abundance of false positives and false negatives results. DNA contamination

could take place due to the environmental condition of the experiments done, or from the utensils used to prepare the reaction mix, or from the living cell contaminations wiped out from surfaces and lab equipment.

3.2. Implications of the Present Study

In the present study, respiratory samples of human origin were analyzed for the detection of respiratory pathogens by the Multiplex Real Time PCR method. The identification of pathogens also helped determining the number of viruses and bacteria present in the samples. In cohort to other studies carried out so far, our results also showed the incidence of mixed infection with viruses or bacteria [28,29]. Onset of pneumonia with such mixed infections may occur up to 45% cases in children and 15% of infections in adults. Unfortunately, the appropriate causative agent can not be identified even in 50% cases despite careful testing [31-33]. Due to lack of the employment of sensitive methods, developing countries meet such diagnosis problems.

In Bangladesh, while an array of diagnostic research has been carried out with parasitic and opportunistic infections, and with tuberculosis, proper investigation in pneumonia diagnosis with a strategy of establishing molecular diagnostic methods is still infant [30-33]. However, the effectiveness of the molecular method lies over the sensitivity and specificity although its shortfall tickles from the cost involvement in resource poor settings.

4. Conclusion

In developed countries, Multiplex real-time PCR pathogen detection assays are currently being used for the detection of the presence of potentially harmful pathogens in high risk environments [34,35]. Our study imparts the ability to accurately detect a spectrum of clinically significant viruses and bacteria rapidly enough to affect patient management and initiation of infection control measures. A quantitative as well as simultaneous identification of the predominant mixed population of microorganisms including Influenza B, S. pneumoniae, equine arteritis virus, para influenza virus and the respiratory syncytial viruses A and B revealed the emerging need of the method endorsement besides the traditional diagnostic methods currently being used. However, the utilization of molecular testing, particularly highly multiplexed tests in routine patient management will depend on the cost/benefit ratio.

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