Available online at http://www.journalijdr.com

ISSN: 2230-9926

IJDR

International Journal of
DEVELOPMENT RESEARCH

International Journal of Development Research
Vol. 4, Issue, 2, pp. 355-358, February, 2014

Full Length Research Article

MOLECULAR DIAGNOSTIC VALUE OF PNEUMOCOCCAL PNEUMONIA AMONG EGYPTIAN CHILDREN

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ARTICLE INFO

Article History:
Received 12th November, 2013
Received in revised form
02nd December, 2013
Accepted 27th January, 2014
Published online 21st February, 2014

Key words:
PCR;
autolysin gene;
Streptococcal pneumoniae

ABSTRACT

Pneumonia is the most common invasive pneumococcal disease; however, only a small portion of cases can be confirmed by conventional techniques. The present study aimed at comparing the molecular detection of *Streptococcal pneumoniae* by the standard blood culture in community-acquired pneumonia among Egyptian children. Standard blood culture of *Streptococcal pneumoniae* and sequence-specific PCR amplification of autolysin gene were performed. There was a statistically significant increase in PCR diagnosed cases than that diagnosed by blood culture with higher PCR sensitivity. Also, there was a statistically significant decrease in culture diagnosed cases among those receiving empirical antibiotic treatment prior sampling. This study concluded that molecular detection of *Streptococcal pneumoniae* may be more accurate and rapid in diagnosis of pediatric community-acquired pneumonia below five years than the standard blood culture.

INTRODUCTION

Pneumococcus is an important human pathogen that causes pneumonia which is the most common invasive pneumococcal disease (Johansson *et al*., 2010). It accounts for up to 4 million deaths a year in developing countries in children below five years (Saad *et al*., 2013); however, microbiologic confirmation of pneumococci is difficult. Diagnosis of pneumococcal Community-Acquired Pneumonia (CAP) has been made through conventional culture but yields are low (Mandell *et al*., 2007) and available only 24–36 hours after blood collection, a delay that will be reflected in patient management (Charkaluk *et al*., 2006). In the absence of a reliable and rapid diagnosis, empirical use of broad-spectrum antibiotics exposes the patients to adverse medication effects, and emergence of multidrug-resistant strains (Black *et al*., 2010). Work on molecular diagnosis has been ongoing (Resti *et al*., 2010). Autolysin (*lyt A*) gene, encoding autolysin, a well characterized virulence marker of pneumococci (Berry *et al*., 1989), has restricted allelic variations. The *lyt A* gene therefore makes an ideal target for specific identification of pneumococci in clinical and epidemiological studies (Llull *et al*., 2006). So this study was conducted to compare the molecular detection of *lyt A* gene of pneumococci with the standard blood culture as a method for the etiological diagnosis of pneumococcal CAP among Egyptian children below five years.

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MATERIALS AND METHODS

This study included 40 CAP cases admitted at the Department of Pediatrics, Benha University Hospital (with positive C-reactive protein and high total leukocyte count). Immune-compromised or lung cancer children were excluded. Approval of the Ethical Committee of the Faculty of Medicine, Benha University and informed consents from parents of children were obtained. The standard blood culture and molecular detection of pneumococci were done. The patients were exposed to full history taking, complete clinical examination and routine laboratory tests (complete blood count and CRP), culture and PCR diagnosis of pneumonia.

Sampling

Venous blood sample (5ml) was taken from each child; 3ml directly inserted into blood culture bottle, incubated at 37°C

and 2ml immediately put in sterile vaccutainer tube with EDTA, mixed, aliquoted (one part was used for CBC and the other was stored at -80°C for PCR).

Blood culture

Standard blood culture, aerobic and anaerobically subcultures were performed. S. pneumoniae were identified by accurate observation of its morphology, α-hemolysis of blood agar, catalase negativity, optochin susceptibility and bile solubility (Kellogg *et al*., 2001).

PCR amplification of lyt A gene

- Genomic DNA extraction from 400ul blood (with EDTA) using Genomic DNA purification kits (Fermentas, Germany) following the manufacturer instructions. The ratio between the absorbance values (optical densities) at 260 and 280 nm gives an estimate of DNA purity as reported by Mohamed *et al*. (2010).

- PCR amplification of autolysin gene using single-plex PCR in G-storm thermal cycler (England) with initial denaturation at 95ºC (3min) then 35 cycles of denaturation, annealing and extension (95ºC 1min, 55ºC 1min and 72ºC 2min respectively), then final extension at 72ºC for 10min. Primers were provided by (Operon, inc Huntsville, Alabama Germany); forward primer 5′-CAA CCG TAC AGA ATG AAG CGG-3′ and reverse primer 5′-TTA TTC GTG CAA TAC TCG TGC G-3′. Green PCR master mix 2x (Fermentas, Germany) was used in 50µl reaction; [25µl Taq PCR master mix 2x, 2.5µl forward primer, 2.5µl reverse primer, 5µl DNA template and nuclease free water up to 50µl]. Concentration of amplified DNA product by spectrophotometer= 50 ug/ml X A260 X dilution factor (Wilfinger *et al*., 1997). Bands were suspected to be seen at 308 bp.

- Gel electrophoresis of amplified products; 10µL reaction mixture and 5µL of 1000 base pair ladder were separated on 2% agarose gel with 0.3ug/ml ethidium bromide. The bands were visualized using UV transilluminator (254 nm) and imaged with a digital camera 8 mega pixel. The image was analyzed by computer software (Alpha In no Tech Gel Documentation System).

Statistical analysis

Qualitative data were expressed in number and percent. Appropriate statistical tests using SPSS version 8 were performed (Cohen's k test and Fisher exact probability test). ROC curve analysis was used to determine the diagnostic power of each test. Results were statistically significant at $p < 0.05$.

RESULTS

Standard blood culture and genomic PCR amplification of pneumococcal *lyt A* gene were done to determine the etiological diagnosis of pediatric CAP cases (♂:♀ 21:19). The study showed a statistically significant increase in CAP cases diagnosed by PCR (13/40) ($p< 0.05$), than that diagnosed by culture (3/40) (Table 1). None of the children negative for pneumococcal infection by PCR was positive by standard blood culture (Table 1). PCR specificity was 100% compared to culture (Table 2). Area under the curve (AUC) for PCR diagnosis was 0.88 (76.5% sensitivity, 100% positive predictive value and 85.2% negative predictive value) in respect to 0.62 for culture AUC (23.5% sensitivity, 100% positive predictive value and 63.9% negative predictive value) (Table 2 and Fig.2). PCR diagnosed CAP cases were statistically increased in previous asthmatic patients ($p<0.05$) (Table 3). Culture positive cases were statistically decreased by empirical antibiotic treatment before diagnosis ($p<0.01$); however, PCR results were not affected (Table 3).

Table 1. Standard blood culture and PCR in diagnosis of pediatric community-acquired pneumonia

			Culture		Total	Cohen's k	P
			-ve	+ve			
PCR	-ve	n.	27	0	27	0.288	<0.01*
		%	100.0%	0.0%	100.0%		
	+ve	n.	10	3	13		
		%	76.9%	23.1%	100.0%		
Total		n.	37	3	40		
		%	92.5%	7.5%	100.0%		

-ve: negative, +ve: positive, n.: number, *P<0.05 is significant

Table 2. Comparison of different tests in diagnosis of pediatric community-acquired pneumonia

Variable	Sensitivity	Specificity	PPV	NPV	AUC	95% CI	P
PCR (+ve)	76.5%	100.0%	100.0%	85.2%	0.88	0.76-1.00	<0.001*
Culture (+ve)	23.5%	100.0%	100.0%	63.9%	0.62	0.44-0.80	>0.05
TLC (13.7 x10^3/µl)	58.8%	69.6%	58.8%	69.6%	0.67	0.50-0.84	>0.05
CRP (72 ng/ml)	82.4%	34.8%	48.3%	72.7%	0.59	0.41-0.76	>0.05

+ve: positive, *: P<0.05 is significant, NPV: negative predictive value, PPV: positive predictive value, AUC: area under the curve, CI: confidence interval, TLC: total leukocyte count, CRP: capsular reactive protein

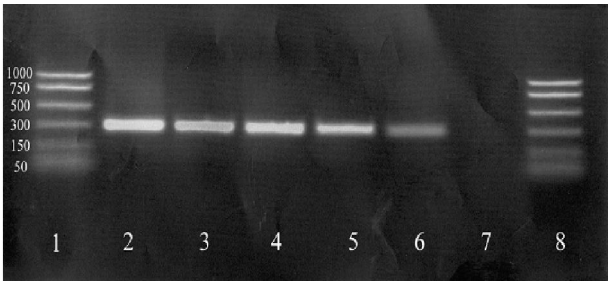

Fig. 1. Gel electrophoresis; DNA ladder in lanes 1 and 8 (its molecular weight 1000 base pairs), pneumococcal *lyt A* gene amplified products in lanes 2-6 (bands appeared at 308 bp) and a negative control in lane 7.

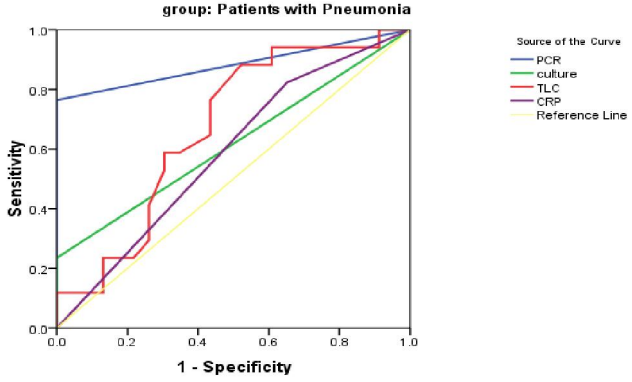

Fig. 2. ROC curve analysis for PCR, culture, total leukocyte count and C-reactive protein in diagnosis of pneumococcal pneumonia

Table 3. Percentage of cases with history of asthma, antibiotic treatment prior diagnosis and clinical diagnosis in relation to PCR and standard blood culture results among pneumonia cases

			PCR		Total	Fisher's P	Culture		Total	Fisher's P
			-ve	+ve			-ve	+ve		
History of asthma	-ve	n.	25	8	33	0. 027*	31	2	33	>0.05
		%	92.6%	61.5%	82.5%		83.8%	66.7%	82.5%	
	+ve	n.	2	5	7		6	1	7	
		%	7.4%	38.5%	17.5%		16.2%	33.5%	17.5%	
Antibiotic treatment prior diagnosis	-ve	n.	3	4	7	0.19	4	3	7	<0.01*
		%	11.1%	30.8%	17.5%		10.8%	100%	17.5%	
	+ve	n.	24	9	33		33	0	33	
		%	88.9%	69.2%	82.5%		89.2%	0%	82.5%	
Clinically	Lobar pneumonia	n.	3	3	6		5	1	6	
	Broncho-pneumonia	%	50%	50%	15%	0.37	83.3%	16.7%	15%	>0.05
		n.	24	10	34		32	2	34	
		%	70.6%	29.4%	85%		94.1%	5.9%	85%	

-ve: negative, +ve: positive, n.: number,*P<0.05 is significant

DISCUSSION

The progressive emergence of antibiotic resistance in S. pneumoniae requires rapid and accurate diagnosis (Llull *et al*., 2006). Our work team chose CAP in children below five years because of more frequent episodes of respiratory tract infection with consequent disruption of normal lung structure and function, in addition to lack of immunization against S. pneumoniae in Egypt (Mishaan *et al*., 2005). In the present study; there is a statistical significant increase in the number of cases diagnosed by PCR compared to culture (P<0.05) (Table 1). Target gene amplification has enabled the detection of low numbers of pathogens in clinical samples (Fredriksson-Ahomaa and Korkeala, 2003). There is also enhanced PCR specificity (100%) that is the same for culture (Table 1), as PCR-based detection depends on amplification of species-specific genes that are unique to pneumococci. Molecular assays targeting the autolysin gene do not cross-react with other streptococcal species (Blaschke, 2011). This study also shows enhanced PCR sensitivity (76.5%) that was diminished for culture (23.5%) (Table 2), this could be explained as PCR detection is not diminished with non viable organisms (McAvin *et al*., 2001); however, culture is affected by the tendency of S. pneumoniae to autolyze when reaching the stationary phase of growth, antibiotic treatment prior to specimen collection and difficulty with adequate specimen collection (Blaschke, 2011). These results agreed with Peters *et al*., (2009) who reported superiority of *lyt A* PCR over blood culture in pretreated patients.

The present study showed a statistically significant increase in PCR–diagnosed cases among patients with history of bronchial asthma (p<0.05) (Table 3); choking episodes that follow asthmatic attacks may predispose to aspiration pneumonia especially in 6-months to 3-years age (Patria and Esposito, 2013). In this study, there was a statistically significant decrease in culture results among those receiving antibiotics prior sampling; the only 3 cases who were culture positive were not receiving antibiotics prior sampling (p<0.01) (Table 3); so blood culture sensitivity was decreased by impaired micro-organism viability as explained by Ramirez and Melo-Cristino (2010). However, Zhang et al. (1995) attributed the decrease in culture results to sample preparation defects.

Conclusion

Molecular detection of S. pneumoniae from blood of children with CAP may be more valuable in early rapid etiological diagnosis of pneumococcal pneumonia. PCR was not affected by antibiotic treatment prior diagnosis or susceptibility to autolyze, but culture was.

Acknowledgement

Deep thanks to Prof. Amal Idris Ali (Head and Chairman of Molecular Biology Unit) and to Dr. Ahmed Abd-Alla (Clinical Pathology Department), Benha Faculty of Medicine (www.fac.bu.edu.eg).

REFERENCES

Johansson N., Kalin M., Tiveljung-Lindell A., Giske C.G. and Hedlund J. 2010 Etiology of community-acquired pneumonia: increased microbiological yield with new diagnostic methods. Clin. Infect. Dis., 50: 202–9.

Saad K., Mohamed S.A. and Metwalley K.A. 2013 Recurrent/Persistent Pneumonia among Children in Upper Egypt. Mediterr. J. Hematol. Infect. Dis., 5: e2013028.

Mandell L.A., Wunderink R.G., Anzueto A., Bartlett J.G., Campbell G.D., Dean N.C., Dowell S.F., File T.M. Jr., Musher D.M., Niederman M.S., Torres A. and Whitney C.G. 2007 Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin. Infect. Dis., 44: S27–72.

Charkaluk M.L., Kalach N., Mvogo H., Dehecq E., Magentie H., Raymond J., Gendrel D., Kremp O. and Decoster A. 2006 Assessment of rapid urinary antigen detection by an immunochromatographic test for diagnosis of pneumococcal infection in children. Diagn. Microbiol. Infect. Dis., 55: 89- 94.

Black R.E., Cousens S., Johnson H.L., Lawn J.E., Rudan I., Bassani D.G., Jha P., Campbell H., Walker C.F., Cibulskis R., Eisele T., Liu L. and Mathers C. 2010 Global, regional and national causes of child mortality in 2008: a systemic analysis. Lancet, 375: 1969-87.

Resti M., Moriondo M., Cortimiglia M., Indolfi G., Canessa C., Becciolini L., Bartolini E., de Benedictis F.M., de Martino M. and Azzari C. 2010 Community-acquired bacteremic pneumococcal pneumonia in children: diagnosis and serotyping by real-time polymerase chain reaction using blood samples. Clin. Infect. Dis., 51:1042–9.

Berry A.M., Lock R.A., Hansman D. and Paton J.C. 1989 Contribution of autolysin to virulence of streptococcus pneumonia. Infec. Immun., 57: 2324- 30.

Llull D., López R. and García E. 2006 Characteristic Signatures of the lytA Gene Provide a Basis for Rapid and Reliable Diagnosis of Streptococcus pneumoniae Infections. J. Clin. Microbiol., 44: 1250–6.

Kellogg J.A., Bankert D.A., Elder C.J., Gibbs J.L. and Smith M.C. 2001 Identification of Streptococcus pneumoniae revisited. J. Clin. Microbiol., 39: 3373–5.

Mohamed M.A., El Moaty M.A., El Kholy A.F., Mohamed S.A. and Ali A.I. 2010 Thrombophilic gene mutations in women with repeated spontaneous miscarriage. Genetic Test. Mol. Biomarkers, 14: 593-7.

Wilfinger W.W., Mackey K. and Chomczynski P. 1997 Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques, 22: 474-176.

Mishaan A.M., Mason E.O. Jr., Martinez-Aguilar G., Hammerman W., Propst J.J., Lupski J.R., Stankiewicz P., Kaplan S.L. and Hulten K. 2005 Emergence of a predominant clone of community-acquired Staphylococcus aureus among children in Houston. *Pediat. Infect. Dis. J.,* 24: 201- 6.

Fredriksson-Ahomaa M. and Korkeala H. 2003 Low occurrence of pathogenic Yersinia enterocolitica in clinical, food and environmental samples: a methodological problem. *Clin. Microbiol. Rev*., 16: 220–9.

Blaschke A.J. 2011 Interpreting Assays for the Detection of Streptococcus pneumoniae. Clin. Infect. Dis., 52: S331–S337.

McAvin J.C., Reilly P.A., Roudabush R.M., Barnes W.J., Salmen A., Jackson G.W., Beninga K.K., Astorga A., McCleskey F.K., Huff W.B., Niemeyer D., Lohman K.L. 2001 Sensitive and specific method for rapid identification of streptococcus pneumonia using real-time fluorescence PCR. J. Clin. Microbiol., 39: 3446- 51.

Peters R.P., de Boer R.F., Schuurman T., Gierveld S., Kooistra-Smid M., van Agtmael M.A., Vandenbroucke-Grauls C.M., Persoons M.C., Savelkoul P.H. 2009 Streptococcus pneumonia DNA load in blood as a marker of infection in patients with community-acquired pneumonia. J. Clin. Microbiol., 47: 3308- 12.

Patria M.F. and Esposito S. 2013 Recurrent Lower Respiratory Tract Infections in Children: A Practical Approach to Diagnosis. Ped. Res. Rev., 14: 53–60.

Ramirez M. and Melo-Cristino J. 2010 Expanding the Diagnosis of Pediatric Bacteremic Pneumococcal Pneumonia from Blood Cultures to Molecular Methods: Advantages and Caveats. *Clini. Inf. Dis. J*., 51: 1050- 2.

Zhang Y., Isaacman D.J., Wadowsky R.M., Rydquist-White J., Post J.C. and Ehrlich G.D. 1995 Detection of Streptococcus pneumoniae in whole blood by PCR. *J. Clin. Microbiol*., 33: 596-601.

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