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Hyaluronatelyase production by *Streptococcus pneumoniae* isolated from patients and carriers

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Abstract. Hyaluronatelyase produced by various microorganisms are capable of degrading hyaluronic acid in connective tissues and initiating the spread of infection by opening an access for the pathogen into host tissues. The present study attempts to determine the distribution of hyaluronatelyase-producing *Streptococcus pneumoniae* among invasive, non-invasive and carriage isolates, and correlate it with the clinical sources, year of isolation, colonial morphology and their serotypes. A total of 100 isolates from various clinical samples were selected and screened for hyaluronatelyase production and presence of the encoding *SpnHyl* gene. All isolates possessed *SpnHyl* gene. Ninety-six isolates including 34 carriage isolates were positive for production of hyaluronatelyase. Four hyaluronatelyase-negative isolates were from blood (2 isolates) and sputum (2 isolates). No significant association was detected among hyaluronatelyase production and bacterial characteristics except for colonial morphology (p = 0.040). High percentages of hyaluronatelyase production in these isolates suggest their possible role as human pathogens.

Streptococcus pneumoniae is a colonizer in the human upper respiratory tract. It is also capable of causing infections ranging from mild to severe sinusitis, otitis media, pneumonia, bacteraemia and meningitis. Young children, the elderly and immuno compromised individuals such as HIV infected patients are at increased risk of these infections (Poll & Opal, 2009). Apart from the well-recognized virulence factor polysaccharide capsule, *S. pneumoniae* has other components including surface proteins, toxins and secreted enzymes which contribute to pathogeneses. One of these virulence factor is hyaluronatelyase. It is a group of hyaluronidase enzyme which degrades hyaluronan, a major component of extracellular matrix in vertebrate connective tissues (Jedrzejas, 2001). This ubiquitous component is distributed in many parts of human body such as umbilical cord, synovial fluid, dermis and vitreous body(Fraser *et al.*, 1997). Hyaluronan is hydrophilic and because of this characteristic, it is able to bind to large number of water molecules thus controlling the viscosity level of the tissue matrix (Delpech *et al.*, 1997).

Hyaluronatelyase has been detected in many species of bacteria including *Streptococcus*, *Staphylococcus*, *Peptostreptococcus*, *Clostridium*, *Streptomyces* and *Propionibacterium* (Hynes & Walton, 2000). Streptococcal species which produce hyaluronatelyase include *S. pneumoniae*, *S. viridans* and streptococcus group A, B, C and G.

S. pneumoniae hyaluronatelyase was implicated as one of the factors contributing either to colonization or its penetration into host tissues. Degradation of hyaluronan by

this enzyme decreases tissue viscosity which results in increasing connective tissue permeability. This allows the bacteria or its secreted products to get into the tissue and spread to other parts of the body. In addition, depolymerisation of hyaluronan to unsaturated dissacharide products provides nutrients and energy sources for bacterial growth and multiplication in the host (Marion *et al.*, 2012).

Function, structure and mechanism of action of the hyaluronatelyase are well documented (Li *et al.*, 2000; Nukui *et al.*, 2003). However, little published data is available regarding distribution of hyaluronatelyase producing pneumococcal strains in relation to bacterial characteristics such as clinical source of isolation, colonial morphology or serotypes. The present study attempts to address these issues.

One hundred pneumococcal isolates from cultures of different individuals compiled during earlier studies (1989 to 2012) were included. Thirty-four isolates were derived from invasive sites, 32 from non-invasive sites and 34 from anterior nares of healthy individuals. Invasive site-isolates were defined as strains isolated from sterile site such as blood, pleural (PF) and cerebrospinal fluid (CSF) of hospitalized patients. Non-invasive site-isolates were derived from non-sterile sites of hospitalized patients including sputum, pus, throat, ear and eye. Carriage isolates were defined as pneumococcal isolates taken from anterior nares of healthy individuals. They were obtained from healthy children less than 5 years old who attended day care centers (Yatim *et al.*, 2013). All isolates were grown on 5% sheep blood agar (Isolac, Malaysia) and incubated at 37ºC with 5% CO_2 for 16 to 18 hours. Re-identification of pneumococcal isolates was done through observation of hemolytic activity and optochin susceptibility on blood agar plate, solubility in bile solution and Gram staining.

Bacterial DNA was extracted from pure cultures on blood agar plates incubated for 16 to 18 hours using commercial extraction kit (GeneAll, Korea) and served as templates for detection of pneumolysin (*ply*), autolysin (*lytA*), *SpnHyl* gene and PCR-based serotyping. Amplification of specific fragment in *ply* and *lytA* gene, as molecular identification of *S. pneumoniae* was done as described elsewhere (Salo *et al.*, 1995; Nagai *et al.*, 2001). Determination of serotypes was done using serotype- or serogroup-specific primers and PCR parameters as previously published (Pai *et al.*, 2006; Dias *et al.*, 2007; Pimenta *et al.*, 2009; Carvalho *et al.*, 2010). Primers grouping for multiplex PCR amplification were done following the strategy as described in Shakrin *et al.* (2013).

Detection of *SpnHyl* gene was performed using a set of self-designed primers with sequence F:5'-TGCCTGGTACAACAGA GACG-3' and R:5'-GCACGATCCACGTTAG GAAT-3' which amplified 562 base pairs sequences in length. A total of 20µl of PCR reaction mixture was prepared by adding 2µl of 10X PCR buffer, 1µl of 25mM $MgCl_2$, 0.4µl of 10mM dNTPs, 0.4µl of 10µM forward and reverse primers, 0.2µl of 5U/µl *Taq* polymerase (Vivantis, Malaysia), 3µl of bacterial DNA and 12.6µl distilled water. Amplification was carried out through a cycle of initial denaturation at 95ºC for 2 minutes followed by 30 cycles of denaturation at 95ºC for 20 seconds, annealing at 68ºC for 10 seconds and extension at 72ºC for 30 seconds. The amplification was ended with final extension at 72ºC for 4 minutes and electrophoresed on 2% agarose gel at 90V for 50 minutes.

Screening of hyaluronatelyase-positive *S. pneumoniae* was performed according to diffusion hyaluronic acid agar plate method as described earlier (Smith & Willet, 1968). The basic medium for diffusion agar plate, Brain Heart Infusion (BHI) was prepared by dissolving 5.2g of BHI agar (Difco, USA) in 100ml distilled water and autoclaved at 121ºC for 15 minutes before cooling down at 46ºC. Aqueous hyaluronic acid was prepared at a concentration of 2mg/ml and sterilized with 0.22µm membrane filter. Aqueous bovine serum albumin (BSA) was prepared at 5% concentration and filtered. All media was prepared at pH 6.8±0.2. Hyaluronic acid solution was added into cooled BHI agar to give final concentration of 400µg/ml. BSA

solution was added into the agar with constant stirring to give final concentration of 1%. The agar mixture was poured into plates with 3 to 4mm in depth. The plate was stored at 4°C prior to use. Two to three pure colonies of 16 to 18 hours incubated pneumococcal isolates on blood agar were streaked on hyaluronic acid agar plate and incubated at 37°C with 5% CO_2 for 16 to 18 hours. Screening test was duplicated in different plate. One ml of 2N acetic acid was flooded onto the plate and left at room temperature for 10 minutes. Clearing zone surrounding the bacterial colonies was indicative of hyaluronatelyase-producing *S. pneumoniae*. Commercial bovine testicular hyaluronidase (Sigma, USA) was applied as positive control. Association of hyaluronatelyase production and bacterial characteristics were statistically tested through Fisher's Exact Test. p value <0.05 was deemed as significant value.

Majority of pneumococcal invasive site-isolates were derived from blood (n=30, 88.2%), and remaining isolates from CSF (3, 8.8%) and pleural fluid (1, 2.9%). Non-invasive site-isolates were mostly obtained from sputum (16, 50%), followed by throat (10, 31.3%), eye (3, 9.4%), pus (2, 6.3%) and ear (1, 3.1%). All thirty-four carriage isolates were obtained from anterior nares swabs of healthy children attending various day care centres(Yatim *et al.*, 2013). Thirty-eight isolates were retrieved from culture collection from 1989 to 2001 and 62 isolates from current collection from 2010 to 2012. Nine isolates appeared as mucoid smooth glistening colonies on blood agar plate and remaining 91 isolates showed non-mucoid rough colonies. Seventeen serogroups or serotypes were identified from all isolates regardless of their defined group where serotype 23F, 19F and 6A/B showed highest types (n=25, 19 and 16, respectively). All isolates showed amplification of *ply*, *lytA* and *SpnHyl* gene.

Screening of hyaluronatelyase activity was done through visualization of clear zones surrounding the bacterial colonies with turbid background of the agar. Non degraded substrate in hyaluronic acid agar was conjugated with bovine serum albumin under acidic condition leaving clear zone of degraded substrate by hyaluronatelyase enzyme secreted by the isolates (Smith & Willet, 1968). Ninety-six isolates showed clear zone surrounding the colonies on the hyaluronic acid agar plate flooded with acetic acid indicating positive production of hyaluronatelyase. Four isolates did not show any clearing zone indicating negative hyaluronatelyase where two of them were isolated from invasive site (blood) and two from non-invasive sites (sputum). All carriage isolates produced hyaluronatelyase. High production of hyaluronatelyase by colonizing pneumococci might be caused by the nature of the human airway structure which has low amount of carbohydrate to provide nutrient for bacterial growth. It may stimulate pneumococci to produce hyaluronatelyase to degrade hyaluronic acid in airway epithelial cells into disaccharides which could be used as nutrient sources for their growth (Marion *et al.*, 2012).

Hyaluronatelyase is produced by various bacterial species (Hynes & Walton, 2000; Makris *et al.*, 2004). The protein structure and sequences of pneumococcal hyaluronatelyase are highly similar with other streptococcal species such as *S. pyogenes*, *S. agalactiae* and *Staphylococcus aureus* (Hynes *et al.*, 2000). High activity of hyaluronatelyase in *S. agalactiae* was not related to specific serotypes or source of isolation (Benchetrit *et al.*, 1987). In *S. suis*, hyaluronatelyase is highly produced by strains of serotype 3 and 7 but less by strains of serotype 9 and 1 (King *et al.*, 2004). The present study attempts to correlate hyaluronatelyase production in relation to bacterial characteristics in pneumococcus. No significant association between hyaluronatelyase and bacterial characteristics was found except for colonial morphology (p = 0.04, <0.05) (Table 1).

Not all isolates produced the enzyme in the presence of its encoding gene. Mutagenesis studies in *SpnHyl* gene have reported as the causes of decreased or absence of enzymatic activity in *S. pneumonia* (Li *et al.*, 2000; Jedrzejas *et al.*, 2002). Nucleotide variation analysis of *SpnHyl* gene in our isolates is currently under investigation.

Table 1. Source, serotypes and hyaluronate lyase detection in pneumococcal isolates

Bacterial characteristics		Hyaluronatelyase, n(%)		*p* value*
		Positive	Negative	
Isolate group	Invasive	32 (94.1)	2 (5.9)	0.462
	Non-invasive	30 (93.8)	2 (6.2)	
	Carriage	34 (100.0)	0	
Sites of isolation	Blood	28 (93.3)	2 (6.7)	0.460
	CSF	3 (100.0)	0	
	PF	1 (100.0)	0	
	Sputum	14 (87.5)	2 (12.5)	
	Throat	10 (100.0)	0	
	Eye	3 (100.0)	0	
	Pus	2 (100.0)	0	
	Ear	1 (100.0)	0	
	Anterior nares	34 (100.0)	0	
Colony morphology	Mucoid	7 (77.8)	2 (22.2)	0.040
	Non-mucoid	89 (97.8)	2 (2.2)	
Year of isolation	1989 – 2001	36 (94.7)	2 (5.3)	0.491
	2010 – 2012	60 (96.8)	2 (3.2)	
Serotypes or serogroups	1	4 (100.0)	0	0.626
	11A	3 (100.0)	0	
	12F	1 (100.0)	0	
	14	4 (100.0)	0	
	15B/C	6 (100.0)	0	
	17F	1 (100.0)	0	
	18A/B/C	2 (100.0)	0	
	19A	8 (100.0)	0	
	19F	18 (94.7)	1 (5.3)	
	20	1 (100.0)	0	
	23A	2 (100.0)	0	
	23F	23 (92.0)	2 (8.0)	
	3	1 (50.0)	1 (50.0)	
	4	2 (100.0)	0	
	6A/B	16 (100.0)	0	
	7A/F	3 (100.0)	0	
	9N	1 (100.0)	0	

*Fisher's Exact Test

Although hyaluronatelyase is one of the many virulence factors associated with *S. pneumoniae* its role requires further evaluation employing genetic and molecular methods.

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