Molecular Identification of Hyaluronic Acid Genes of *Streptococcus pyogenes*

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Abstract

The genes responsible for hyaluronic acid (HA) production (*has A,has B* and *has C* genes) of eight local *S. pyogenes* isolates were identified using polymerase chain reaction(PCR). It was found that all isolates possessed *has A and has B* genes whereas *has* C gene was found in three isolates of *S. pyogenes* . This study supports the opinion that *has A and has B* genes are required for direct HA biosynthesis, while *has* C gene is not required for HA production.
Keywords : *Streptococcus pyogenes*, hyaluronic acid, PCR, *has A, B and C genes* .

الخلاصة

شخصت الجينات المسؤولة عن انتاج حامض الهاليرونك (جينات *has A* و*has B* و *has C*) لثمانية عزلات محلية للمسبحيات القيحية باستخدام تفاعل البلمرة المتسلسل (PCR) . وجد ان كل العزلات تمتلك جينات *has A* و*has B* بينما جين *has C* وجد في ثلاثة عزلات من المسحيات القيحية . تدعم هذه الدراسة فكرة ان *has A* و*has B* ضرورية للتصنيع المباشر لحامض الهاليرونك بينما الجين *has C* غير ضروري لانتاج حامض الهاليرونك.
الكلمات المفتاحية : المسبحيات القيحية , حامض الهاليرونك , PCR ، جينات *has A* و *B* و*C*.

Introduction

Hyaluronic acid is a biopolysacharide with high molecular weight ascertained by Meyer and Palmer (1934) in the vitreous of Cow's eyes. HA is considered as the simplest entities of a group of substances known as glycosaminoglycans. It is characterized by the non covalently companion with a core protein not synthesized via the Golgi pathway and the only non-sulfated one (Cowman and Matsuoka, 2005 ; Choi *et al.*, 2014). Since the hyaluronan polymer created in animals and the aforesaid bacteria are identical, the host immune defense is not induced to reject the pathogenic bacteria reversely to other bacteria with a different extracellular capsule. Intriguingly, the nonimmunogenic and noninflammatory properties of bacterial hyaluronan benefit mammals currently too, since bacterial hyaluronan forms an excellent source for medical grade hyaluronan (Boeriu *et al.*, 2013). In the human body, HA exists in the salt hyaluronate form and is present at high concentrations in the skin, umbilical cord and vitreous humor (Liu *et al*., 2011). Primarily, HA is not just found in the extracellular matrix and pericellular matrix, but has also been shown to persist intracellularly (Necas *et al.*, 2008). Previously, HA was extracted from rooster combs, while now it is mainly produced by microbial fermentation with less production costs and lower environmental pollution. Industrially, HA has been successfully produced on a large scale using the *Streptococcus sp*. as the main producer (Liu *et al*., 2011). Many studies have revealed that hyaluronic acid is not only considered as a lubricant with dermatologic and ophthalmologic applications but also can be employed in

accomplishing the drug release for instances, in anesthesia prolongation in bones and joints (Goldenheim *et al.*,2001), arthropathy treatment (Suzuki *et al.*, 2002), chemotherapeutic agents in surgical implants , drug release in dental caries, controlled antigen release for immunotherapy , and contact lenses, and as a copolymer with anti-thrombotic properties in vascular applications .The main dermatological diligence of hyaluronic acid is developing soft tissues by intradermic injections to compensate skin problems driven by wrinkles, scars, lip enlargement or other defects (Rosa *et al.*, 2012). In 1993 the first gene encoding the enzyme responsible for hyaluronan synthesis denoted HA synthase or HAS was recognized as beginning from group A streptococci (DeAngelis *et al.*, 1993a). The gene responsible for the coding of enzymes that direct the biosynthesis of the group A streptococcal hyaluronic acid capsule is *has ABC* gene cluster (Ashbaugh *et al*., 1998). Hyaluronan synthase the gene product of *hasA*,catalyzes the assembly of hyaluronic acid from N-acetylglucosamine and glucuronic acid (DeAngelis *et al.*, 1993b; Dougherty and Van de Rijn, 1994). *has B* encodes UDP-glucose dehydrogenase which configurates glucuronic acid from UDP-glucose (Dougherty and van de Rijn, 1993). *has C* encodes UDP-glucose pyrophosphorylase which forms P-glucose from UTP and glucose-1-phosphate (Crater *et al.*, 1995). Although the *has ABC* genes are adjacent and form an operon (Crater and Van de Rijn, 1995) complementation experiments with both group A hemolytic Streptococci (GAS) and heterologous bacteria have suggested that *has C* may not be required for capsule synthesis (DeAngelis *et al.*, 1993a).This study aimed to identify the genes responsible for the control of hyaluronic acid production of local *S. pyogenes* isolates.

Materials and Methods

Bacterial Isolates

Eight isolates of *S. pyogenes* were isolated and identified depending on traditional methods as described by MacFaddin (2000) , in addition to the use of the strepto- system 9R according to the manufacture instructions. These isolates were used for further experirments.

DNA Extraction

Chromosomal DNA was extracted from fresh overnight culture grown in a nutrient broth at 37 ºC using a GenElute™ Bacterial Genomic DNA Kit (Sigma-USA).

PCR Amplification

Singleplex was used in this study, 5 µl of master mix (GeneAll, Korea), 3µl of each primer , 5 µl of template DNA and 4 µl of dieionized water mixed in 20 µl of total reaction volume. Amplification reactions were performed in an automated thermocycler (Clever Scientific, UK) apparatus.

Table 1: Sequences, product size and references of primers used for PCR amplification of *S. pyogenes* isolates

Genes	Primer sequence 5'----------→ 3'	Product Size(bp)	Reference
hasA	P050F 5 –GTTATCGTTCACCGTTCCC-3 P052R 5-TACGTGTTCCCCATTCCG-3		(Ward *et al.*, 2001)
hasB	JL7F 5- ATATTCTTCCCTCTAAAGTTGATATGA-3 JL8R 5- GAATGTCAATAACATCTTTGATAGCAC-3		(Levin and Wessels, 1998)
has C	D-34aF 5-GCACATATGACCAAAGTCAGAAAAG-3 D-37aR5-GCAGGATCCAAGTACCAACACATTACTTTG-3	925	(Crater *et al.*, 1995)

Thermal cycling conditions

Amplification was performed in an automated thermocycler, programmed as mentioned in table 2.

Table 2. Thermal cycling conditions of *has A, has B* and *has C* genes of *S. pyogenes* isolates

Gene	Cycle No.	Temperature (ºC)	Time(min.)
has A	1	94	2
	30	94	1
		47.8	0.5
		72	2
	1	72	5
		4	Hold
has B	1	94	5
	32	94	0.5
		59	0.75
		72	1
	1	72	5
		4	Hold
has C	1	95	2
	32	95	0.5
		59	0.5
		72	1.7
	1	72	5
		4	Hold

Agarose gel electrophoresis

The electrophoresis was performed according to Sambrook and Russel (2001). The PCR products were run on horizontal agarose gel (0.8%) stained with ethidium bromide for 90 min. and 70 volt. The DNA bands were photographed by Gel documentation system (Biomera Co. Germany).

Results and Discussion

The molecular identifications of *has A, has B and has C genes* of S. *pyogenes* were studied. It was found that the primer pair hasA-F and hasA-R corresponding to *has A* gene of *S. pyogenes* generated amplicon with approximately 880 bp from one isolate, while the rest isolates produced two amplicons at about 800 bp and 880 bp (Fig. 1)

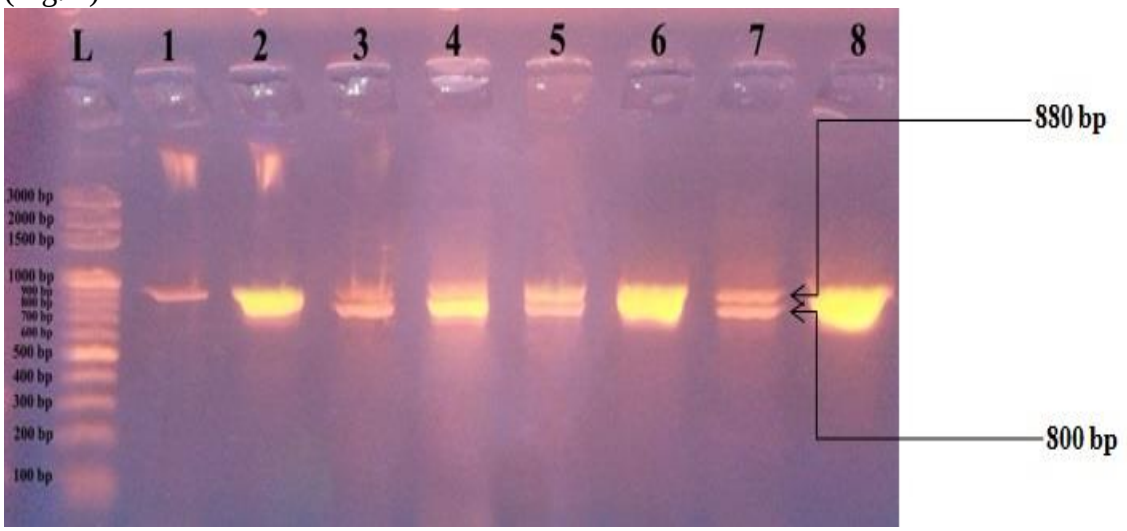

Fig 1: Electrophorasis of the amplified product of *has A* gene on o.8 % agarose gel. Lane L: DNA ladder, containing molecular size standards (bp), lanes 1to 8 : *S.pyogenes* isolates.

The result of amplification of *has B* gene revealed that all *S. pyogenes* isolates generated products at about 925 bp (Fig. 2).

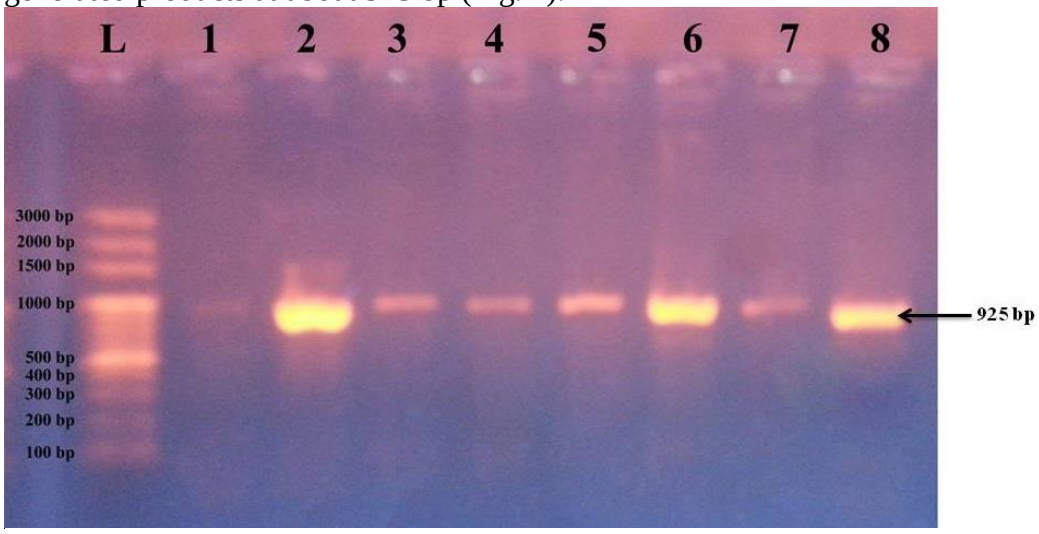

Fig 2: Electrophorasis of the amplified product of *has B* gene on o.8 % agarose gel. Lane L: DNA ladder, containing molecular size standards (bp), lanes 1to 8 : *S.pyogenes* isolates 1 to 8,respectively.

Amplification of the *has C* gene of *S. pyogenes* isolates with primer designated D-34a and D37a showed DNA samples of only three *S.pyogenes* isolates generated a PCR product of the expected size (925bp) (Fig. 3).

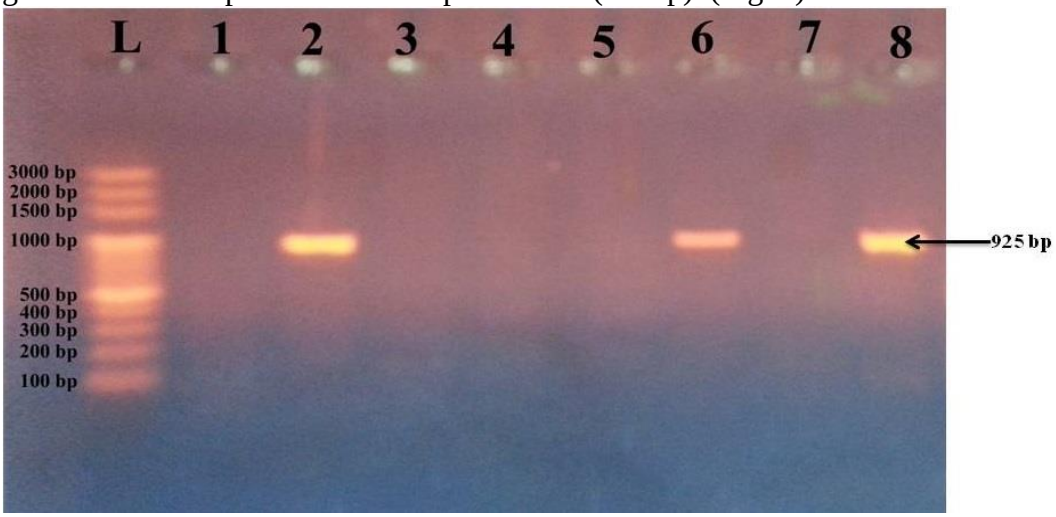

Fig 3: Electrophorasis of the amplified product of *has C* gene on o.8 % agarose gel. Lane L: DNA ladder, containing molecular size standards (bp), lanes 1to 8 : *S.pyogenes* isolates 1 to 8,respectively.

This result agreed with Crater *et al.*(1995) study, that showed *has C* was demonstrated to consist of 915 bp. The *has C* gene product, UDP-glucose pyrophosphorylase is not required for hyaluronic acid synthesis indicating that an alternative source of UDP-glucose is available for capsule production (Ashbaugh, *et al*., 1998). In *S. pyogenes* the capsule biosynthetic operon is comprised of the *hasA*, *hasB*, and *hasC* genes, which encode hyaluronic acid synthetase, UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase, respectively. The third gene in the operon *has C* is not required for capsule production due to the existence of another gene of UDP glucose pyrophosphorylase in the genome (Kang *et al.,* 2012). *has A* and *hasB* are the only exogenous genes required to direct HA biosynthesis in most bacteria due to the presence of one of the sugar nucleotide precursors of HA, UDP-GlcNAc, which is necessary for cell wall formation. In cells that make both UDP-GlcNAc and UDP-GlcA only HA synthase, the gene product of *has A* is needed to polymerize the HA polysaccharide (DeAngelis *et al.,* 1993). DeAngelis *et al.* (1993) find that a putative *has C* gene is not required for HA capsule biosynthesis.

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