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Fusion of HPV L1 into *Shigella* surface IcsA: A new approach in developing live attenuated *Shigella*-HPV vaccine

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ABSTRACT

Despite the success of L1 virus-like particles (VLPs) vaccines in prevention of high-risk human papillomavirus (HPV) infection and cervical cancer, extraordinary high cost for the complete vaccination has impeded widespread use of the vaccine in resource-poor countries, where cervical cancers impose greater challenge. Presentation of HPV L1 protein by attenuated pathogenic bacteria through natural infection provides a promising low-cost and convenient alternative. Here, we describe the construction and characterization of attenuated L1-expressing *Shigella* vaccine candidate, by fusion of L1 into the autotransporter of *Shigella sonnei*, IcsA, an essential virulence factor responsible for actin-based motility. The functional α domain of IcsA was replaced by codon-optimized L1 gene with independent open reading frames (ORFs) facilitated by suicide vector pJCB12. The L1 gene was stabilized in the genome of recombinant *S. sonnei* with protein expression and assembly of VLPs in the bacterial cytoplasm. Through conjunctival route vaccination in guinea pigs, L1-containing *S. sonnei* was able to elicit specific immune response to HPV16 L1 VLP as well as bacterial antigens. The results demonstrated the feasibility of the novel stratagem to develop prophylactic *Shigella*-HPV vaccines.

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1. Introduction

Infection of high-risk types of human papillomavirus (HPVs) is the major cause of cervical cancer, the second leading malignancy of women worldwide (Bosch et al., 2002; Garland et al., 2007; Snoeck, 2006). Currently, the L1 virus-like particles (VLPs) vaccines have been used in prevention of HPV infection and related neoplastic disease (Campo and Roden, 2010; de Borba et al., 2009; Garland et al., 2007; Kirnbauer et al., 1992; Rose et al., 1993). The two licensed L1 VLP vaccines (Gardasil, Merck & Co., Inc., and Cervarix, GlaxoSmithKline), produced in eukaryotic system, are against two dominant oncogenic HPV genotypes, HPV16 and HPV18, which together account for about 70% of cervical cancers (Campo and Roden, 2010; Harper et al., 2004; Koutsky et al., 2002). However, high cost of these HPV vaccines hinders the application of these vaccines in resource-poor countries which have 80% cervical cancer cases (Campo and Roden, 2010). Thus, cost-effective new vaccines will be the key to widespread vaccination for control of cervical cancer.

The virus capsid L1 protein can be expressed and self-assembled into functional pentamers and even VLPs in bacteria (Baud et al., 2004a; Fraillery et al., 2007; Mustafa et al., 2009; Nardelli-Haefliger et al., 1997; Revaz et al., 2001; Yuan et al., 2001), which offer a cheaper and practical alternative to VLP-based vaccines. Previous studies have demonstrated the feasibility of L1 expression and self-assembly in attenuated pathogenic bacteria like *Salmonella* as well as *Shigella* from plasmid vectors. Immunization of small animals by these recombinant bacteria could stimulate specific immune responses to HPV VLPs (Baud et al., 2004a; Baud et al., 2004b; Fraillery et al., 2007; Yang et al., 2005). However, the instability of the plasmid vectors *in vivo* without antibiotics selection has become obstacle in the further improvement of these vaccine candidates (Nardelli-Haefliger et al., 1997; Revaz et al., 2001). For improving the stability of the plasmid vector and optimized expression of L1, attempts have been made for codon optimization (Baud et al., 2004b) and change of antibiotic marker in *Salmonella* (Fraillery et al., 2007). However, we reasoned that integration of the L1 gene into the bacterial genome can covercome the requriement of antibiotic selection and improve stability of the viral gene in bacterial host.

Shigella causes diarrhea by invading and destroying colorectum epithelium. The bacterium is able to proliferate inside the cell cytosol and to spread intra- and inter-cellularly via the action of IcsA, a surface protein abundantly expressed in all virulent *Shigella* strains (Goldberg et al., 1994). Disruption of IcsA leads to loss of actin-based motility, resulting in markedly reduced virulence in

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humans and animal models (Lett et al., 1989; Schroeder and Hilbi, 2008). In this study, we replaced part of the IcsA coding sequence with the HPV16 L1 gene, which achieved high expression of the viral VLP and attenuation of the bacterial virulence. The resultant vaccine strains were potent in eliciting immune responses to HPV as well as to *Shigella sonnei* via conjunctiva route in guinea pigs.

2. Methods and materials

2.1. Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* and *S. sonnei* strains were routinely cultured on L-agar and L-agar containing Congo red (0.1%), respectively, at 37 °C. Antibiotics (Sigma) were used as follows: streptomycin 100 μg/ml; chloramphenicol 20 μg/ml.

2.2. DNA manipulations

Chromosomal and plasmid DNA was extracted for various *in vitro* manipulations. Oligonucleotides were ordered from Sigma and listed in Table 2. Polymerase chain reaction (PCR) was performed by Gene Engine (Bio-Rad) using Hotstar mixture (Qiagen). Bacterial transformation by electroporation or heatshock was performed as described previously (Calvin and Hanawalt, 1988).

2.3. Construction of icsA-HPV16 L1 transcriptional gene fusion

The coding sequence of residues 105–506 (Charles et al., 2001) of *S. sonnei* IcsA (http://www.ncbi.nlm.nih.gov/nuccore/NC_007385.1) was replaced with entire HPV16 L1 gene (http://www.ncbi.nlm.nih.gov/nuccore/JX897004.1). To construct the gene fusion, 3 fragments were PCR amplified: the first fragment included 312 bp of the *icsA* 5'-end coding sequence and 550 bp upstream non-coding sequence with a stop codon at the 5'-end by primers P1F and P1R; the second fragment was the entire coding sequence of HPV16 L1 gene by primers P2F and P2R with independent SD sequence 11 bp in front of the L1 start codon at the 5'-end and a stop codon at the 3'-end; the third fragment included the coding sequence of *icsA*, bp 1519–3306, by primers P3F and P3R. All the fragments were sequentially ligated and cloned into pUC19 using In-Fusion™ Advantage PCR Cloning Kit (Clontech) to generate pUC19-*icsA*-L1. In the resultant construct, the transcription of L1 gene was driven by the *icsA* promoter and its translation was initiated via the independent SD sequence. Since the L1 gene was not in-frame with either side of the *icsA* coding sequence and therefore it could produce intact L1 protein.

Table 2
Oligonucleotides used in this study.

Name	Sequence	Target
P1F	CGGTACCCGGGGATCTAGTTATGTTTGATGTCTGCA	*icsA*
P1R	CTG TTTCCTGTAATTTAAAGT TCTAGATGCATGAGAGG	*icsA* (outframe)
P2F	AATTACAGGAAACAGGTATGAGCCTGTGGCTGCCCA	HPV16 L1 (outframe)
P2R	ACTTCATTTAACAGCTTCCTCTTCTTCCTC	HPV16 L1 (outframe)
P3F	GCTGTTAAATGAAGTACTATTCTGGCAGATAAT	*icsA* (outframe)
P3R	CGACTCTAGAGGATCTCAGAAGGTATATTTCACAC	*icsA*
SP1F	GGTGCATCCCTGTTGCTCG	*icsA*
SP1R	TGGGCACCAGGATCTTGTTG	HPV16 L1
SP2F	ACACCTTCTGGGAGGTGAAC	HPV16 L1
SP2R	CCAGCGGTACGTGTCATAGC	*icsA*
P19F	GATCCTCTAGAGTCGACCTGC	Linearized pUC19
P19R	GATCCCCGGGTACCGAGCT	Linearized pUC19

The gene fusion construct (Fig. 1A) was subcloned into the suicide vector pJCB12, to give rise to pJCB12-*icsA*-L1. The subclone was maintained in *E. coli* SM10λ*pir*, which supports the replication of R6K origin plasmids and allows the expression of the plasmid-borne *tra* genes for conjugation.

2.4. Construction of the Shigella-HPV vaccine strain

This was achieved via conjugation. A streptomycin resistant derivative SS86St^r of a wildtype *S. sonnei* strain SS86 was isolated as recipient strain and the *E. coli* SM10λ*pir*/pJCB12-*icsA*-L1 as the donor. As illustrated in Fig. 1B, a single crossover was achieved by selecting chloramphenicol and a double crossover was achieved by sucrose selection as described by Turner (Turner et al. (2006), Turner et al. (2001)). The resultant colonies were analysed by PCR-electrophoresis (Fig. 1C as well as DNA-sequencing of the PCR products to confirm the second crossover and the presence of the constructed gene fusions.

2.5. Cell infection and fluorescent imaging

Gentamycin-killing assay was performed as previously described (Lucchini et al., 2005). Briefly, HEp-2 (ATCC CCL-23) cells were cultured in Dulbecco's minimal essential medium (DMEM) (Sigma–Aldrich) containing 10% fetal bovine serum with 5% CO_2 at 37 °C and seeded in 24-well plates and cultured to ~80% confluence. *S. sonnei* strains from the mid-exponential phase were added to the cells at an MOI (*m*ultiplicity *o*f *i*nfection) of 10 (for CFU

Table 1
Bacterial strains and plasmids.

Name	Relevant genotype, phenotype, and description	Source/reference[a]
Strains		
DH5α		Promega
SM10λ*pir*	*pir tra*RP4	SI
SS86	*S. sonnei* (wild-type *S. sonnei*)	SRL
SS86St^r	Strepmycin resistant derivative of SS86	This work
SS86St^r/L1	*S. sonnei* with HPV16 L1 infused with *icsA* in transcriptional-fusion ORFs	This work
SS86Δ*icsA*/p322L1	*S. sonnei* with deletion in *icsA* harboring plasmid carrying L1 viral gene	
Plasmids		
pUC19	2690 bp plasmid for infusion PCR fragments, *ori*UC, *cat*, *lacZ*, Linearized vector generated by PCR	Clontech
pUC19-*icsA*-L1	4168-bp fragment, including *icsA* gene with HPV16 L1 in transcriptional-fusion ORFs, in pUC19	This work
pJCB12	oriR6 K mobRP4 sacBcat	SI
pJCB12-*icsA*-L1	4168-bp fragment, including *icsA* gene with HPV16 L1 in transcriptional-fusion ORFs, in pJCB12	This work

[a] SI, provided by Dr. Arthur K. Turner from The Wellcome Trust Sanger Institute, Cambridge, UK; SRL, provided by Scottish *Salmonella* and *Shigella* Reference Laboratory, Glasgow, UK.

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