A bioshield against influenza virus infection by commensal bacteria secreting antiviral peptide

JD Huang *, BJ Zheng, KY Yuen

KEY MESSAGE

A commensal bacterial strain was engineered to express and secret antiviral peptide against influenza A virus.

Hong Kong Med J 2016;22(Suppl 4):S13-5
RFCID project number: 08070592

[1] **JD Huang**, [2] **BJ Zheng**, [2] **KY Yuen**

[1] *Department of Biochemistry, The University of Hong Kong*
[2] *Department of Microbiology, The University of Hong Kong*

* Principal applicant and corresponding author: jdhuang@hku.hk

Introduction

Avian influenza is caused by influenza viruses such as the influenza A virus. In 1997 in Hong Kong, the avian influenza viruses were found to cross the species barrier to infect human beings and cause respiratory illness and death. H5N1 viruses later re-emerged in Asia with human cases of infection, of which more than 50% were fatal.

We proposed to genetically engineer naturally occurring bacteria to provide protection against influenza A virus infection by secreting a peptide that interferes with viral attachment. Introduction and colonisation of these genetically modified bacteria in the oral mucosa and respiratory tract would provide a natural bioshield for uninfected individuals and protection against influenza A virus.

Methods

This study was conducted from December 2008 to May 2011. In order for the bioshield to be successful, there should be (1) continuous expression and secretion of a potent antiviral peptide at a high level; (2) colonisation of the genetically modified microbes on the mucosal surface of the respiratory tract for prolonged periods to block infection by influenza A virus; and (3) no pathology in the colonised host caused by the genetically engineered bacteria. Similar strategies have been used to protect animals against HIV,[1] *Candida albicans*,[2] and *Streptococcus mutans*.[3] The technology and 'bio-bricks' necessary for building an anti-H5N1 bacterium strain are all available as follows:

(1) Bacterial strains that colonise the respiratory tract: unlike immunisation in which the bacteria need to exist only for a limited period, antiviral peptide has to be present continuously in the secretions of the upper airway when the patient is challenged by the influenza virus. This can only happen if the bacteria producing the antiviral peptide form a normal commensal bacterial strain that has a survival advantage in the pharynx. Gram-positive bacteria such as *Streptococcus* and *Lactobacilli* have a distinct advantage. *Streptococcus gordonii* has the best track record.[4] *S gordonii* is a commensal bacterium commonly found on healthy human oral mucosa, the upper respiratory tract, and in dental plaque with a high colonising ability. Although associated with dental caries, it is less virulent than *Streptococcus mutans* but does not lose the ability to colonise the oropharynx continuously. It forms commensal microflora in humans without causing any pathology.

(2) Peptide with anti-influenza virus function: a 20-amino-acid peptide derived from the signal sequence of fibroblast growth factor 4 has exhibited broad-spectrum antiviral activity against influenza viruses including the highly pathogenic H5N1 subtype.[5] This peptide prevents viral infection by binding to the viral haemagglutinin protein to block attachment of the virus to the cellular receptor *in vivo* and *in vitro*. A 100% survival was observed when BALB/c mice were infected with H5N1 A/Hong Kong/156/97 strain pre-treated with 2 mM of this peptide. In addition to the preventive effect, this peptide showed promising therapeutic value. When BALB/c mice were first infected with A/Hong Kong/156/97 and then given 2 mM of the peptide intranasally, there was 100% survival at 7 dpi, compared with 0% survival without treatment. Importantly, mice that received this peptide alone exhibited no toxicity.[5]

In this project, the pLEX secreting expression system was first tested with green fluorescent protein (GFP). Then an antiviral peptide gene was cloned into the pLEX vector, transforming the resulting plasmid into *S gordonii*. The expression and secretion of antiviral peptide in the culture supernatant was examined by western blot and dot blot. The protective function of the peptide

was evaluated *in vitro* by the haemagglutination inhibition (HI) assay. To verify the protection *in vivo*, mice were challenged with lethal influenza virus in the presence or absence of the engineered bacteria.

Results

The efficiency of pLEX expression system in *S gordonii* GP204 stain was verified by GFP report protein (Figs 1 and 2). Nevertheless, the expression and secretion of the target antiviral peptide (EB) could not be detected in the supernatant of *S gordonii* GP204 culture by western blot or dot blot. Therefore, a series of pLEX-xEB clones was constructed to optimise expression. With the optimised EB expression system, secreted EB still could not be detected by western blot in the supernatant. The protective effect of EB expression was evaluated *in vitro* using HI assay. EB peptides had been secreted into the culture supernatant. The secreted EB blocked influenza virus and inhibited binding of the influenza virus to turkey red blood cells (Fig 3).

The protective effect of EB-expressing *S gordonii* against influenza virus in mice was evaluated. First, the colonisation of *S gordonii* in mice was evaluated. Four weeks after inoculation, 75% of mice were still colonised with *S gordonii*. To evaluate the protective effect, mice colonised with *S gordonii* were challenged with lethal influenza virus. There was no significant difference in survival rate between the group colonised with *S gordonii* harbouring pLEX-4EB and the control group. Further optimisation strategies of the expression system for increasing EB expression *in vivo* are necessary.

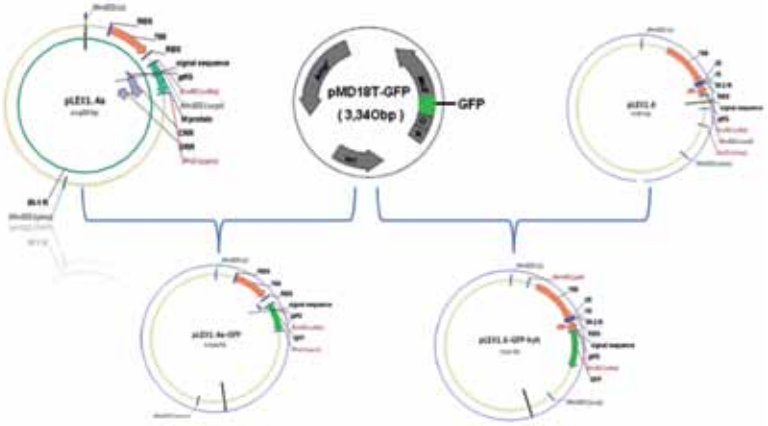

FIG 1. Construction of pLEX1.4a/1.6-GFP

Discussion

S gordonii GP204 has been extensively studied and used to surface display or secrete various functional proteins.[6] We successfully verified the efficiency of the pLEX expression system in *S gordonii* GP204 stain by GFP reporter protein. Nevertheless, when detecting EB expression in *S gordonii* GP204, there were unexpected difficulties. Unlike GFP protein, in the supernatant and cells of recombinant bacteria, EB expression could not be detected by western blot or dot blot. This might have been because: (1) EB expression was too low to be detected. It is possible that the promoter activity of *gtfG* in pLEX may be insufficient to secrete detectable amounts of small peptide. (2) Detection method was inappropriate. The dot-blot or western blot assay may not be sensitive enough to detect such a small peptide (~2 kDa). (3) It is possible that only relatively large proteins can be secreted in this system; it is

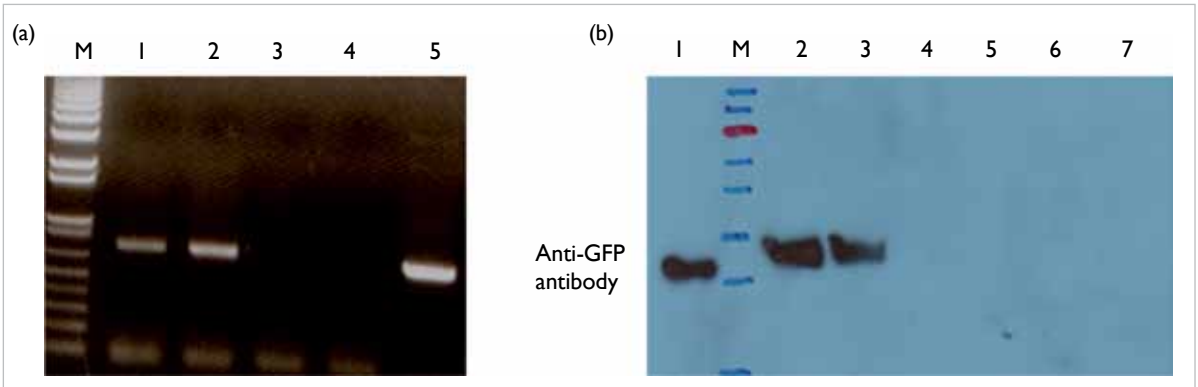

FIG 2. (a) Verification of transformation by bacteria liquid PCR: 1 denotes culture of pLEX1.4a-GFP transformed *S gordonii* GP204, 2 Culture of pLEX1.6-GFP transformed *S gordonii* GP204, 3 culture of blank *S gordonii* GP204 as negative control, 4 pLEX1.6 plasmid as negative control, and 5 pLEX1.6-GFP plasmid as positive control. (b) Verification of GFP expression in culture supernatant and cells by western blot: 1 denotes cell 1 of 7207-GFP as positive control, 2 supernatant of *S gordonii* GP204 transformed with pLEX1.4a-GFP, 3 supernatant of *S gordonii* GP204 transformed with pLEX1.6-GFP, 4 supernatant of blank *S gordonii* GP204 as negative control, 5 cells of *S gordonii* GP204 transformed with pLEX1.4a-GFP, 6 cells of *S gordonii* GP204 transformed with pLEX1.6-GFP, and 7 cells of blank *S gordonii* GP204 as negative control.

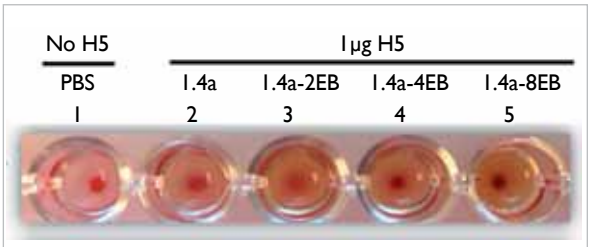

FIG 3. Haemagglutination inhibition assay

not known if small peptides (ie 20 aa) can also be secreted by this strategy.

Therefore, our optimisation strategy was to multiply the sequence of EB. We constructed a series of pLEX-xEB-his clones to optimise the secreting expression. Likewise, western blot results detected no EB-his expression in culture supernatant. We then decided to detect the presence of EB by its protective function. This was performed *in vitro* by HI assay. The HI assay result indicated that EB peptides were likely secreted into the culture supernatant, as only the bacteria harbouring EB expressing plasmids can block influenza virus and inhibited influenza virus from binding to turkey red blood cells. The bacteria that contained the empty vector failed to block viral binding. Hence, we further evaluated the protection against influenza virus in mice. There was no significant difference in survival rate between the pLEX-4EB group and GP204 control group. We suspect that the expression of antiviral peptide was too low to completely block the influenza virus. It is possible that the amount of inoculated bacteria that could secrete antiviral peptide was insufficient, or that the efficiency of the pLEX system *in vivo* was not as high as necessary. Another possible reason was that EB peptides might have been broken down by *S gordonii*. A minimal and optimal sequence of EB peptide has been reported to improve the antiviral efficacy and decrease the synthesis costs.[7] Nonetheless, it is unknown whether this minimal epitope will be better expressed in *S gordonii*. It is also very difficult to detect and evaluate the secreting expression of small epitopes in the supernatant. Although EB is not toxic to either HeLa or Vero cells at 100 μM, toxicity of the peptide to *S gordonii* cannot be excluded. In future, we will commercially synthesise EB peptide and evaluate its toxicity at various concentrations with *S gordonii*.

Overall, we successfully verified the efficiency of pLEX expression system in *S gordonii* GP204 stain by GFP reporter protein and constructed a series of engineered commensal bacteria strains. Nonetheless, to build an effective anti-influenza bioshield, there remain a few hurdles to be overcome: (1) It is difficult to obtain high level secreting expression of EB peptide both *in vivo* and *in vitro* thus resulting in no effective protection. (2) The plasmid containing the EB peptide sequence is unstable and can be easily lost *in vivo* and may contribute to low peptide expression. (3) *S gordonii* colonisation in mice is not stable and needs multiple inoculations supplied with appropriate antibiotics.

Although the challenge experiments did not provide efficient protection, the supernatant of the engineered bacteria did show evidence of viral inhibition. This offers hope that the strategy may work by improving the expression and colonisation efficiency. If successful, the novel bioshield can be inoculated by simple combination with nutritious foods or encapsulated. This will be more convenient and acceptable to use, and easier to administer. The bioshield can be self-administered and eliminates the use of a hypodermic needle. A bioshield against avian influenza viruses is easy to manufacture.

Acknowledgement

This study was supported by the Research Fund for the Control of Infectious Diseases, Food and Health Bureau, Hong Kong SAR Government (#08070592).

References

1. Chang TL, Chang CH, Simpson DA, et al. Inhibition of HIV infectivity by a natural human isolate of Lactobacillus jensenii engineered to express functional two-domain CD4. Proc Natl Acad Sci U S A 2003;100:11672-7.
2. Beninati C, Oggioni MR, Boccanera M, et al. Therapy of mucosal candidiasis by expression of an anti-idiotype in human commensal bacteria. Nat Biotechnol 2000;18:1060-4.
3. Krüger C, Hu Y, Pan Q, et al. In situ delivery of passive immunity by lactobacilli producing single-chain antibodies. Nat Biotechnol 2002;20:702-6.
4. Sharma A, Sojar HT, Hruby DE, Kuramitsu HK, Genco RJ. Secretion of Porphyromonas gingivalis fimbrillin polypeptides by recombinant Streptococcus gordonii. Biochem Biophys Res Commun 1997;238:313-6.
5. Jones JC, Turpin EA, Bultmann H, Brandt CR, Schultz-Cherry S. Inhibition of influenza virus infection by a novel antiviral peptide that targets viral attachment to cells. J Virol 2006;80:11960-7.
6. Maggi T, Spinosa M, Ricci S, Medaglini D, Pozzi G, Oggioni MR. Genetic engineering of Streptococcus gordonii for the simultaneous display of two heterologous proteins at the bacterial surface. FEMS Microbiol Lett 2002;210:135-41.
7. Jones JC, Settles EW, Brandt CR, Schultz-Cherry S. Identification of the minimal active sequence of an anti-influenza virus peptide. Antimicrob Agents Chemother 2011;55:1810-3.