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BRIEF REPORT

Construction of live-attenuated *Trueperella pyogenes* by antibiotic treatment and sequential passage: methods for vaccine development

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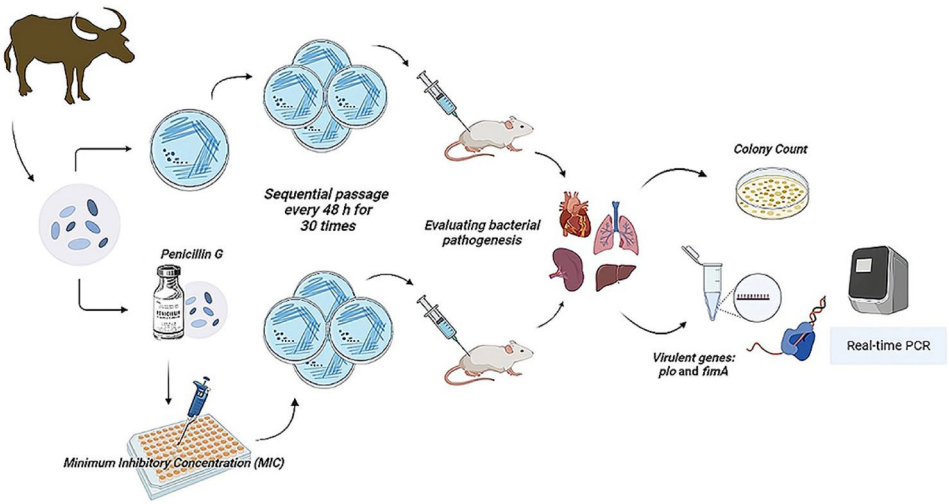
Abstract

Trueperella pyogenes (*T. pyogenes*) is a zoonotic pathogen that is cause a variety of pyogenic diseases in animals. The complex pathogenicity and various virulence factors are important challenges to produce an effective vaccine. According to previous trials, inactivated whole-cell bacteria or recombinant vaccines were unsuccessful in preventing disease. Thus, this study aims to introduce a new vaccine candidate based on a live-attenuated platform. For this purpose, first *T. pyogenes* was subjected to sequential passage (SP) and antibiotic treatment (AT) to lose their pathogenicity. Second, *Plo* and *fimA* expressions as virulence genes were evaluated by qPCR and then mice were challenged with bacteria from SP and AT culture by intraperitoneal route. Compared to the control group (*T. pyogenes*-wild type), *plo* and *fimA* gene expressions were downregulated and vaccinated mice have a normal spleen appearance in contrast to the control group. In addition, there was no significant difference between bacterial count from spleen, liver, heart and peritoneal fluid in vaccinated mice and the control group. In conclusion, this study introduces a new *T. pyogenes* vaccine candidate based on a live-attenuated strategy that mimics natural infection without pathogenicity for further investigation on vaccines against *T. pyogenes* infections.

Graphical abstract



Keywords *Trueperella pyogenes* · Live-attenuated · Vaccine · Virulence genes

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Introduction

Trueperella pyogenes is a commensal and opportunistic pathogen in animals, causing clinical diseases such as pneumonia, mastitis, metritis, abortion, liver abscess and septicemia with economic loss in livestock animals (Rzewuska et al. 2019). The bacterium has been isolated in cattle from the nasopharynx, lymph nodes, uterine secretions, rumen wall and its contents (Rzewuska et al. 2019). Since antibiotic therapy is the main choice for the treatment of bacterial pathogenesis, antibiotic usage in livestock animals has been limited due to the emergence of drug-resistant bacterial species (Ashrafi Tamai et al. 2021b). Thus, the only way to reduce bacterial pathogenesis and prevention of disease is through vaccination. The history of *T. pyogenes* vaccine development goes back 70 years ago. At the first attempt, the bacteria toxoid was used for experimental immunization and challenged animals (Lovell et al. 1950). Inactivated bacteria culture or bacteria alone was also used as a vaccine candidate (Hunter et al. 1990; Jost and Billington 2005). The DNA vaccine is another platform was used *T. pyogenes* vaccine (Huang et al. 2022). Until now, there is no commercial vaccine available (Galán-Relaño et al. 2020b). *T. pyogenes* have several virulence factors including pyolysin (PLO), *fimA* as an adhesive factor, serine proteases with gelatinase and caseinase activity and DNases for invasive to host cells (Rzewuska et al. 2019).

Since PLO is an important virulent factor, this molecule was used for vaccine development. Several studies have used secreted PLO from inactivated *T. pyogenes* supernatant or recombinant PLO that were not suitable vaccines due to the presence of non-PLO components in the supernatant and the toxicity of recombinant PLO, respectively (Lovell et al. 1950; Jost et al. 1999; Machado et al. 2014). On the other hand, using live bacteria to stimulate an immune response against bacterial infection has been a popular vaccine development method for several decades (Detmer and Glenting 2006). Despite the possibility of attenuated bacteria reverting to wild type, live bacterial attenuated vaccines have several advantages, including the ability to mimic a natural infection, natural adjuvant features, induce long-term immunity and the ability to be administrated from natural bacterial invasion routes (Detmer and Glenting 2006). Since no vaccine has been produced based on the live-attenuated bacteria, this study aimed to construct live-attenuated bacteria as a vaccine candidate to reduce the pathogenesis of *T. pyogenes.*

Materials and methods

Bacterial strains and culture conditions

In this study, *T. pyogenes*, number PTCC1925 (Persian Type Culture Collection), isolated from uterus of a water bufalo (*Bubalus bubalis*) in Iran was selected (Ashrafi Tamai et al. 2021a). Lyophilized *T. pyogenes* was cultured in Blood Agar with 7% sheep blood and incubated at 37 °C with 5% CO2 for 48 h, pure colonies with beta hemolysis were selected.

Experiment design

To construct live attenuate *T. pyogenes*, the study was conducted in two ways.

1. *Sequential passage (SP): T. pyogenes* were passages sequentially on a blood agar medium. A passage of bacteria was given every 48 h and performed 30 times.
2. *Antibiotic treatment (AT): T. pyogenes* is susceptible to Penicillin G, to determine the best concentration of Penicillin for the treatment of *T. pyogenes*. First, the minimum inhibitory concentrations (MIC) of Penicillin G were evaluated based on (Galán-Relaño et al. 2020a) and then, the bacteria were cultured at the ½ MIC for 48 h. Second, the bacteria and antibiotic passages sequentially 30 times.

Animal inoculation and samples collection

Animal experiments for in vivo evaluation were performed using 6-week-old female Balb/C mice (20 ± 5 g) (Center of Laboratory Animal, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran) in three experimental groups. Animal experiments were carried out under the guidelines described by the Institutional Animal Care and Use Committee (IACUC). All animal experiments in this study were carried out upon approval by the ethical committee board, Faculty of veterinary medicine, University of Tehran, Tehran, Iran. Mice were given one week before the experiment to adapt to the laboratory condition. Then, 15 mice randomly were divided into 3 groups. Each group contains five mice (SP as the Sequential passage group, AT as the antibiotic treatment group and C as Control group = *T. pyogenes* PTCC1925). All mice were subjected to bacterial injection (1.5×10^8 CFU in 0.2 ml) via IP route. Mice after 72 h were euthanized and the liver, spleen, heart, and peritoneal fluids of each mouse were collected and mixed for further experiments.

Colony count

To evaluate the potential growth of *T. pyogenes* from SP and AT groups in comparison to C group in tissues, 72 h after injection, liver, heart, lung, spleen and peritoneal fluids of the inoculated mice were collected, cut into pieces and mixed with PBS. Mixed and diluted samples were centrifuged, and the supernatant was subjected to colony count (CFU) as described (Ji et al. 2021; Tamai et al. 2022).

Expression of virulent genes

For determining the pathogenicity of bacteria, two genes, *plo* and *fimA*, which are the most important virulent genes and found in all isolates of the *T. pyogenes*, were selected (Rzewuska et al. 2019). Mixed and diluted samples (liver, heart, lung, spleen) were centrifuged, and the supernatant was subjected to RNA extraction and cDNA synthesis CinaClon tissue RNA extraction and cDNA synthesis kits (CinnaGen, Iran) were used according to the manufacturer's instructions. The quality of cDNA templates was characterized using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). cDNA was stored at − 80 °C for qPCR analysis. Primers for the two selected genes and housekeeping gene (*ftsY*) are shown in Table 1 and qPCR was used for the expression of genes. Each sample was repeated three times during a test. The relative expression level was calculated according to the following formula (2^{-dct}) and Fold change was calculated according to Livac method (2^{-ddct}) (Livak and Schmittgen 2001). The obtained number, according to the distance from the number one, showed the order of expression increase or decrease.

Statistical analysis

Two-way ANOVA and Tukey's multiple comparisons test were used to assess significant differences of gene expression between all groups. All values were expressed as mean ± SEM.

Table 1 Primer sequences and PCR conditions

Gene	Primer	Annealing temperature (°C)
Plo	F: TCATCAACAATCCCACGAAGAG	60
	R:TTGCCTCCAGTTGACGCTTT	
fimA	F: CACTACGCTCACCATTCACAAG	60
	R: GCTGTAATCCGCTTTGTCTGTG	
ftsY	F: GGAAGCCGATTGGGAAGAGC	60
	R: TCCGTAGTTGCCTTGACTTTCGT	

Results

Bacterial pathogenicity

To evaluate bacterial pathogenicity, the liver, spleen and heart were collected. The Tissue appearance of the spleen in the control group after 72 h of IP injection of bacteria was bigger than SP and AT groups (Fig. 1). The liver and heart had normal tissue appearance in all groups (data not shown).

Colony count of *T. pyogenes*

The results of this study showed that *T. pyogenes* in SP and AT groups could colonize in liver, heart, lung, spleen and peritoneal fluids of the inoculated mice. However, there is no significant difference between the SP (1.43×10^5) and AT (1.33×10^5) groups in comparison to the control group (1.75×10^5).

Expression of virulent genes

The effect of sequential passage and antibiotic treatment on the expression of *plo* and *fimA* were investigated by qPCR, the results revealed that the *plo* and *fimA* were significantly reduced in SP (Fold change (*plo*) = 0.007, Fold change (*fimA*) = 0.0009) and AT group (Fold change (*plo*) = 0.002, Fold change (*fimA*) = 0.0002) compared to the control group, whereas no significant change was detected between SP and AT group (Fig. 2).

Discussion

T. pyogenes has been identified as an opportunistic infection and pus agent in animals in recent decades, and significant research has revealed that this bacterium affects a wide range of hosts and causes a variety of diseases. Liang et al. found that PLO could induce pyroptosis and IL-1β release

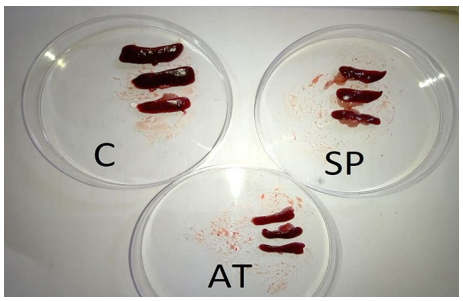

Fig. 1 The morphological comparison of the mice spleen after 72 h of *T. pyogenes* IP injection. The spleen in the control group was bigger than SP and AT groups

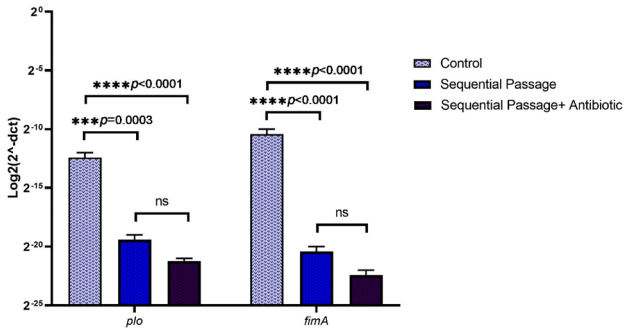

Fig. 2 Quantitative real-time PCR chart; the results showed that sequential passage and antibiotic therapy downregulate the *plo* and *fim*A gene expression compared to the control group, but no significant difference was seen between the SP and AT groups (Log2(relative expression level) has been shown). *ns* non-significant

in murine Macrophages resulting in inflammation (Liang et al. 2022). In a recent study conducted by Meira et al. on dairy cows, they used three vaccine formulations include (FimH, leukotoxin, and pyolysin), and inactivated whole cells (*Escherichia coli, Fusobacterium necrophorum, and Trueperella pyogenes*) or both that reduce puerperal metritis (Meira Jr et al. 2020). However, no effective vaccine has been developed for this disease. The early studies to protect ruminants against *T. pyogenes* infection with whole cells bacteria or culture supernatant were mainly ineffective (Lovell et al. 1950; Hunter et al. 1990; Jost and Billington 2005). In another study, vaccinated pregnant swine with whole cells bacteria treated with phenol, could control the loss of newborn animals (Kostro et al. 2014). Moreover, mice as a reliable model for animal studies were vaccinated with formalin-inactivated recombinant PLO and then challenged with 108 *T. pyogenes* cells by IP route. The results indicate PLO-specific antibody response in vaccinated mice (Jost et al. 1999). In this study, we constructed live-attenuated bacteria as a vaccine candidate to reduce *T. pyogenes* pathogenesis. According to the *plo* and *fimA* gene expression results, sequential passage and antibiotic treatment are effective methods for downregulated virulence genes. In vitro and in vivo research on plo-deficient mutants and recombinant proteins showed that PLO is the main virulence factor of *T. pyogenes* and causes lethal and dermonecrotic effects in mice, rabbits and guinea pigs (Rzewuska et al. 2019). In Zhao et al. study, the *T. pyogenes* strain with higher *plo* gene expression *in vitro* was more virulent for mice than the strain with lower *plo* expression, which was similar to our observation of liver and spleen of ST and AT group in comparison to the control group 11 (Zhao et al. 2013). In addition, Jost et al. reported *plo*-deficient mutant strains lost their hemolytic activity and virulence (Jost et al. 1999).

A function of fimbriae is bacterial adherence to host cells, *T. pyogenes* have five fimbriae including FimA, FimB, FimC, FimE, and FimG (Rzewuska et al. 2019). Zhao et al. investigated the expression of *T. pyogenes* virulence factors, such as FimA and FimC. Their findings revealed that the *fimA* gene was expressed earlier than *fimC* and implying that FimA is the dominant fimbria in *T. pyogenes* (Zhao et al. 2013). Liu et al. speculated that low *fimA* expression can limit the formation of other fimbriae and that increasing this fimbria production could be linked to *T. pyogenes* pathogenicity (Liu et al. 2018). Therefore, the reduction of *T. pyogenes* pathogenicity in ST and AT groups could result in low expression of *plo* and *fimA*. On the other hand, bacterial recovery results from spleen, liver, heart, and peritoneal fluids revealed that antibiotic treatment and the sequential passage do not negative effect on bacteria count, indicating the ability ST and AT groups to mimic natural infection. Live-attenuated vaccines include a form of living pathogenic microbes that has been weakened by sequential passage through a foreign host, culture media, cell culture or embryonic egg for multiple generations. Since attenuated microbes are significantly different from pathogenic forms, they cannot cause disease but can successfully provoke an immune response. They multiply in the host and provide persistent antigenic stimulation for a long time (Yadav et al. 2020). The BCG (Bacillus Calmette-Guérin) vaccine was developed from the Calmette-Guérin strain of *Mycobacterium bovis* and Ty21a vaccine are examples of bacterial live-attenuated vaccines against *Mycobacterium tuberculosis* and *Salmonella typhi* that mimic natural infection in the human host (Yadav et al. 2020).

Conclusion

In conclusion, our study demonstrated antibiotic pressure and sequential passage downregulated the *plo* and *fimA* gene expression and convert wild-type bacteria to attenuated *T. pyogenes*. Importantly, attenuated *T. pyogenes* mimic natural infection in mice models without pathogenicity which is the main feature of live-attenuated vaccines. Thus, this study provides a new vaccine candidate for *T. pyogenes* which needs more research on the immune response.

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Author contributions BB: conceptualization, investigation, methodology. IAT: supervision, conceptualization, writing–original draft, writing–review. TZS: data curation, formal analysis and editing.

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Data availability The data of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest All authors declare that there is not any conflict of interest.

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