

Induction of Antibacterial Activity in the Blowfly Larvae by Natural Infection Model

J Zhuang, J Li, H Wang, Y Yao, P Shan, B Jing, P Han

ABSTRACT

Objective: To investigate the antibacterial activities of blowfly larval induced by natural infection.

Methods: The sterile larvae were mixed in a test tube containing a bacterial *Escherichia coli* (E coli) which was suspended in phosphate buffered saline (PBS), and incubated at 25 ° C for given periods, with sterilized PBS as the control group. Then the haemolymph was collected and tested against *Staphylococcus aureus* (S aureus) and *Pseudomonas aeruginosa* (P aeruginosa), respectively. Diameter ring was recorded to indicate the antibacterial activities.

Results: Infected larvae had better antibacterial capacities than sterile larvae. Antibacterial activities peak appeared at 24 hours and disappeared after 48 hours. The induced haemolymph from the larva possesses stronger antibacterial activity against S aureus than P aeruginosa.

Conclusion: The sterile larva of blowfly, *Lucilia sericata*, antibacterial activities could be induced by a natural infection model.

Keywords: Antibacterial activity, blowfly larvae, natural infection, *Staphylococcus aureus*

Inducción de la Actividad Antibacteriana en Larvas de Mosca Azul Mediante el Modelo de Infección Natural

J Zhuang, J Li, H Wang, Y Yao, P Shan, B Jing, P Han

RESUMEN

Objetivo: Investigar las actividades antibacterianas de la larva de la mosca azul inducida por infección natural.

Métodos: Larvas estériles se mezclaron en un tubo de ensayo que contenía un cultivo bacteriano de *Escherichia coli* (E coli), suspendido en solución salina taponada con fosfato (PBS), e incubado a 25 ° C por períodos dados, con PBS esterilizado como grupo de control. Entonces la hemolinfa fue recogida y sometida a prueba contra *Staphylococcus aureus* y *Pseudomonas Aeruginosa* respectivamente. El anillo del diámetro fue registrado para indicar las actividades antibacterianas.

Resultados: Las larvas infectadas tenían mejores capacidades antibacterianas que las larvas estériles. Las actividades antibacterianas alcanzaron el punto máximo a las 24 horas y desaparecieron después de 48 horas. La hemolinfa inducida de la larva posee una actividad antibacteriana más fuerte contra S aureus que contra P aeruginosa.

Conclusión: Las actividades antibacterianas de la larva de la mosca azul, *Lucilia sericata*, pudieron ser inducidas por un modelo de infección natural.

Palabras clave: Actividad antibacteriana, larvas de mosca azul, infección natural, *Staphylococcus aureus*

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INTRODUCTION

Infected wounds can be treated effectively with maggots of the blowfly *Lucilia sericata*. At present, maggot debridement therapy (MDT) is widely used in the world (1–3). Maggots developing in infected wounds prevented further infection and accelerate wound healing. Maggot debridement therapy was used as a successful treatment of children with severe osteomyelitis by Baer's group (4). Recent studies suggest that, in MDT, the worms not only digest bacteria (5, 6) they inhibit secretion or destruction of biological membrane and promote sterilization. More importantly, the maggots can also secrete antibacterial factor (7–9). Due to clinical success, maggot therapy was used widely in Europe and it was shown that especially in trauma surgery the application of larval therapy was very successful and could even prevent major amputations (10, 11). Supported by these publications, MDT was approved by the US Food and Drug Administration in 2004 [510[k] 33391] (12). Maggot debridement therapy works in three areas, such as debridement, disinfection and stimulation of wound healing (13–18).

However, with the sterile, there is no antibacterial gene expression, which greatly influenced the antimicrobial effects of maggot (19). At present, several methods such as: injection of bacteria in the larval cavity, ultraviolet radiation and ultrasonic techniques build the models. These methods could produce antimicrobial substances. But the natural maggot is in direct contact with the pathogen and develops antibacterial activity by natural infection. In MDT, sterile maggot infection belongs to natural infection (20), antibacterial activity was induced by this method. Therefore, this research has more reference value for clinics. Diabetic foot ulcers and chronic infectious wounds have been considered intractable wound diseases for a long time. Lots of researchers are trying their best to improve the cure results. Only a few kinds of maggots could exhibit antibacterial capacity. In this study, using *E coli* as inducer, from patients with diabetic foot wound are gram positive bacteria *Staphylococcus* and gram negative bacteria *Pseudomonas aeruginosa* (21). As test bacteria,

antibacterial activity was evaluated. The natural infection was induced by maggot.

SUBJECTS AND METHODS

Culture of sterile maggot

Sterile *Lucilia sericata* was preserved fended in our laboratory. The larvae feed on components as: wheat bran 600 g, milk powder 40 g, yeast 8 g, peptone 15 g, rice noodles 10 g, water 1500 mL was mixed, loaded in the feed tank, bound with kraft paper and sterilized with autoclave at 121 °C and then kept in 4 °C refrigerator.

Sterile maggots hatching: 0.1% HgCl₂, 25% ethanol and 0.05% hydrochloric acid to soak the eggs for 5 minutes, the larvae feed on the eggs were inoculated into sterile Petri dish, 25°C dark incubation for 60 hours with sterile conditions.

The test bacteria culture

Escherichia coli, *Staphylococcus aureus* and *Pseudomonas aeruginosa* was preserved in our laboratory. Cultured in LB culture medium to the logarithmic growth phase, was suspended in sterile PBS, and the concentration adjusted to 5 x 10⁸/mL.

Maggot natural infection

Three length of time aseptic maggots 50 were put into 2 mL LB medium with different number of *E coli* bacteria (5 x 10⁸, 1 x 10⁹, 2 x 10⁹) and PBS as control group. It were incubated for 30 minutes at room temperature and then transferred to 25 °C Petri dish which contains larvae were cultured 12 hours, 24 hours, 36 hours, 60 hours, 48 hours then collected maggots lymph.

Maggots lymph collection

Naturally infected maggots were washed repeatedly with cold sterile distilled water five to six times, with cold sterile saline three times. Surface water of parasites was sucked by filter paper. Larval head was cut. Lymph was collected with capillary pipette and transferred to 1.5 mL centrifuge tube. The sample was centrifuged with 4 °C, 6000 r/minute for 15 minutes,

and the supernatant was filtered with 0.45 μm membrane filter and stored at $-80\text{ }^{\circ}\text{C}$ in a refrigerator.

Identification of antimicrobial activity using the plate count method

Thirty-seven degree celsius, 250 r/minute shaking culture two hours and then $5 \times 10^8/\text{mL}$ of *Staphylococcus aureus* or *Pseudomonas aeruginosa* was added to dilute to 2 mL. The bacterial suspension and maggots was diluted, the extract each 100 μL were mixed at $37\text{ }^{\circ}\text{C}$ for six hours, 50 μL culture fluid from each group was spread on LB solid medium, overnight incubation was at $37\text{ }^{\circ}\text{C}$. Calculating the number of colony forming (CFU) in 16 hours, basis on CFU to evaluate the antimicrobial activity of *Staphylococcus aureus* or *Pseudomonas aeruginosa*. Each of the antibacterial tests was repeated for six times.

Statistical analysis

The statistical package for the Social Sciences-10 (SPSS) 10.0 Software Package was used for statistical analysis. Data as mean \pm standard deviation between the two groups were compared using *t*-test, *p*-value < 0.01 was considered statistically significance.

RESULTS

Effect of maggots extract on the growth of *Staphylococcus aureus*

We first tested bacteria *Staphylococcus aureus* to study the antimicrobial activity of natural infection by the number of CFU. Experimental results show that the pre-infection treatment of natural maggots, the maggots extract has high antibacterial activity; sterile maggots extract also has some antibacterial effect, but significantly lower than the extract of infected maggots. Infection, sterility and PBS have significant differences with the control group.

Different doses of *E coli*-induced antimicrobial activity of maggots

Sterile maggots with PBS and 5×10^8 , 2×10^9 a 24-hour incubation of *E coli* extracts maggots were detected *Staphylococcus aureus* and *Pseudomonas aeruginosa* inhibitory effectiveness. For *Staphylococcus aureus*, CFU count 2×10^9 group was significantly less than the sterile control group ($n = 6$, $p < 0.01$), while the number of 5×10^8 and 1×10^9 CFU of *E coli* is less than the sterile control group, but is not statistically significant.

The number of CFU of *Pseudomonas aeruginosa* group showed no significant difference among different groups. *Escherichia coli* infected maggots extract of *Pseudomonas aeruginosa* does not have significant antimicrobial activity (Fig.1).

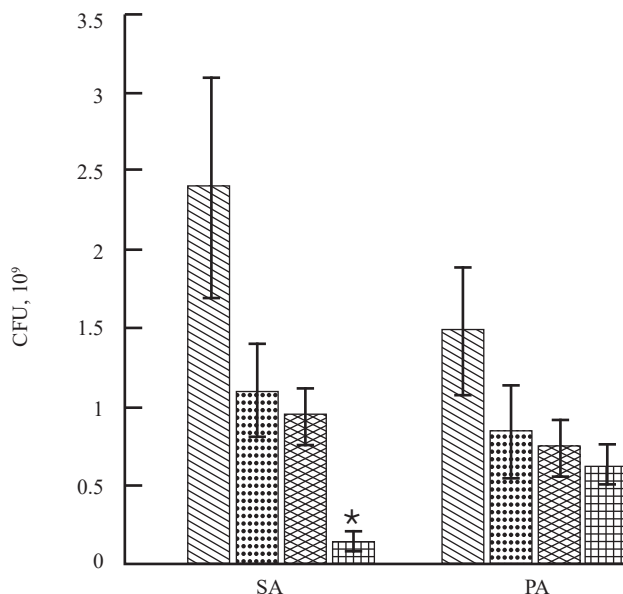


Fig. 1: Different amounts of *E coli*-induced antibacterial activity of maggots.

SA: *Staphylococcus aureus*; PA: *Pseudomonas aeruginosa*; PBS control: 5×10^8 *E coli* infection maggots extract treatment; 1×10^9 *E coli* infection maggots extract treatment; 2×10^9 *E coli* infection maggots extract treatment. * Significant difference

Figure 1 shows: 5×10^8 , 1×10^9 and 2×10^9 group maggots extract in the same manner to the two kinds of bacteria, which have significant antibacterial activity. These results indicate 2×10^9 group and the other two groups on the activation of the immune system have significant differences ($p < 0.05$). 5×10^8 /mL and 1×10^9 /mL samples in both bacteria greater than 2×10^9 *E coli* suspension which can activate the immune system maggots.

Effect of different time of *E coli*-induced antimicrobial activity of maggots

The number of CFU with 2×10^9 *E coli* treated group of *Staphylococcus aureus* at 12-hour, 24-hour and 36 hours was significantly lower than the sterile control group ($n = 6$, $p < 0.01$). The same treatment and the number of CFU 48 hours and 60 hours had less than the trend in the control group, but not statistically significant (Fig. 2).

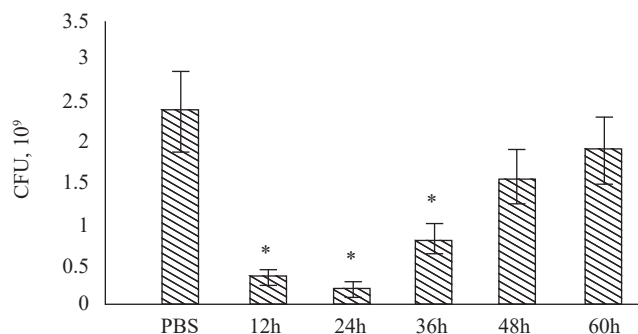


Fig. 2: Effects of antibacterial activity on *Staphylococcus aureus* of *Escherichia coli* infection in different time.

DISCUSSION

The Gram-positive and Gram-negative aerobes from patients' wounds were *S aureus* and *P aeruginosa*, respectively. These are in agreement with foot wounds in diabetes literatures (22–24).

The larvae grown in an infected environment had good antibacterial activity against *S aureus* than sterile larvae. A previous study on antibacterial E/S activity of sterile *L sericata* larvae showed no antibacterial activity against *S aureus* (25).

Pretreatment with a single *S aureus* or *P aeruginosa* could induce the antibacterial activity. This natural infection method activates *Drosophila* larval immunity (20). Although a previous study showed the induction of antibacterial activities in *L sericata* larvae by direct injection of bacteria with an infected needle (26), the natural infection method was considered to better reflect the context of an actual clinical wound. However, our study showed that, no induced larva haemolymph had the antibacterial activity. It showed that the antibacterial substances of immune haemolymph of housefly may be affected by the structure of gene regulation, exogenous induction material (such as infection or parietal damage) quickly contribute to the synthesis of antimicrobial substances, kill bacteria and prevent bacteria from multiplying. At the same time, infection of worms had stronger antibacterial activity than the sterile maggots.

The method of needle injection to infect maggot consume time, UV irradiation and ultrasonic guidance method need special equipment. In this study, using a single *E coli* natural infection of antibacterial activity was induced by worms. Method of natural infection was to be able to better indicate the actual clinical wound environment, so that MDT could be of value.

This study showed that the antibacterial activity induced by *E coli* *Staphylococcus aureus* and *Pseudomonas aeruginosa* was invalid. Previous studies

had shown the worms were more effective against gram-positive bacteria than gram-negative bacteria (9, 10). Some studies showed that when the bacteria amount was small, the worms might directly by the intake of bacteria activate the innate immune system leading to antibacterial activity (5, 6). When the bacteria amount was larger, in order to survive in harmful environment, the immune system was activated to produce new worms antibacterial products (20). In addition, when the maggots hatch, contact with the bacteria after 12–36 hours could induce the antibacterial activity.

CONCLUSION

The methods of natural infection in the environment can induce larvae antibacterial activity. Debridement is an important aspect of the maggot therapy.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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