SHORT COMMUNICATION

Antibacterial properties of larval secretions of the blowfly, *Lucilia sericata*

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Abstract. The antibacterial properties of secretions aseptically collected from larvae of the greenbottle fly *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) were examined. These investigations revealed the presence of small (<1 kDa) antibacterial factor(s) within the larval secretions, active against a range of bacteria. These include the Gram-positive *Staphylococcus aureus*, both methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA), *Streptococcus pyogenes* and to a lesser extent the Gram-negative *Pseudomonas aeruginosa*. These secretions were shown to be highly stable as a freeze-dried preparation and, considering the activity against organisms typically associated with clinical infection, may be a source of novel antibiotic-like compounds that may be used for infection control and in the fight against MRSA.

Key words. *Lucilia sericata*, antibacterial, larval therapy, methicillin-resistant *Staphylococcus aureus*, secretions.

Larva of the greenbottle fly *Lucilia sericata* (Meigen) are increasingly widely used as a fast and effective means of treating necrotic wounds, particularly chronic wounds where conventional treatments have failed (Stoddard *et al.*, 1995; Mumcuoglu *et al.*, 1998, 1999; Thomas *et al.*, 1999a). Indeed larvae from this facultative, myiasis-causing fly are currently the exclusive blowfly species of choice for larval therapy (the application of maggots to wounds) in the U.K.

Larval therapy is certainly not a new technology; reports of blowfly larvae cleansing wounds span many cultures over centuries (Weil *et al.*, 1933; Sherman & Pechter, 1988; Church, 1996). Larval therapy was routinely used with high success rates in the 1930s in response to World War I battlefield surgeon William Baer’s observations and scientific studies (Baer, 1931). The advent of antibiotics in the 1940s, coupled with advances in surgery resulted in a declining use of larval therapy. However, renewed interest in larval therapy has been generated both in hospitals and the wider community over recent years, primarily in response to the search for suitable methods to control chronic wound infection. Wound infection is a universal problem costing the U.K. National Health Service annually an estimated £1 billion (NAO, 2004). This problem is compounded by the widespread occurrence of ‘antibiotic-resistant superbugs’ such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) (Beasley & Hirst, 2004). Despite the widespread, long-term employment of larval therapy, there is surprisingly little known about the mechanisms behind infection control.

The modes of action of ‘wound-healing’ larva may be broadly categorized into three main areas: debridement (Chambers *et al.*, 2003), disinfection and bacterial death (Erdmann & Khalil, 1986), and stimulation of wound granulation and repair (Prete, 1997; Chambers *et al.*, 2003). The end result is clean, healthy tissue at the original wound site. The reduction of bacterial load and disinfection of wounds by maggots was first observed by Dr William Baer, an American orthopaedic surgeon (Baer, 1931). This was demonstrated to be due, at least in part, to bactericidal properties of the gut and/or exo-secretions of *L. sericata* larvae (Simmons, 1935; Pavillard & Wright, 1957). Some researchers have suggested antibacterial action is a result of larval ingestion and gut activity (Robinson & Norwood, 1934; Greenberg, 1968). More recent work, using

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fluorescence microscopy, monitored the death of *Escherichia coli* during larval digestion and demonstrated that bacterial load reduction took place primarily in the midgut of *L. sericata* (Mumcuoglu et al., 2001). Other groups have concentrated on the antimicrobial activity of larval secretions. Phenylacetaldehyde and phenyl acetic acid were isolated from *Proteus mirabilis*, a commensal bacterium from the gut of the screw worm *Cochliomyia hominivorax* (Coquerel), and were shown to be active against both Gram-positive and Gram-negative bacteria (Erdmann et al., 1984; Erdmann & Khalil, 1986). A preliminary study has demonstrated that sterile *L. sericata* secretions exhibit marked antimicrobial activity against liquid cultures of the Gram-positive *Streptococcus* sp., *Staphylococcus aureus* and a clinical strain of MRSA (Thomas et al., 1999b). No attempt was made to characterize the antibacterial activity.

Antimicrobial peptides have been shown to be an essential element of the insect’s innate defence system, providing powerful resistance to a wide range of harmful pathogens and parasites (Boman, 1995, 2000). The majority of those identified have been shown to be inducible, low molecular weight, cationic peptides (Bulet et al., 1999). These compounds have become of considerable interest to the pharmaceutical industry, as they possess a rapid and broad-spectrum of activity with an ability to avoid microbial resistance. Enhancing their therapeutic value yet further, currently no drug resistance or mammalian toxicity has been observed. As such, these peptides appear to be ideal candidates to assist in the fight against the drug-resistant ‘superbug’ strains such as MRSA.

It is possible that such antimicrobial peptides may in part be responsible for the wound-healing properties of secretions from *L. sericata* larva. The object of this study was to characterize further the antimicrobial activity/activities of sterile larvae from *L. sericata* and to see whether these activities exhibited similarities with typical insect antimicrobial peptides.

To obtain larval secretions the following procedures were followed. *Lucilia sericata* blowflies were reared in mesh cages under controlled conditions (25°C; 16:8 h light dark cycle, constant relative humidity, continuous supply of water and sugar). Flies were provided with raw pig liver for oviposition. The resultant eggs were surface-sterilized and seeded onto blood agar plates supplemented with defibrinated horse blood and kanamycin. After 3–4 days incubation at 25°C, 16:8 h light dark cycle, sterile late 2nd/early 3rd stage larvae were aseptically removed from the blood agar plates, washed successively with 70% ethanol and sterile ultrapure water (ddH2O). Elga Ltd, High Wycombe, Buckinghamshire, U.K.), and blotted dry on sterile filter paper. Washed larvae were then transferred to sterile ddH2O at a density of 100 larvae per 10 mL ddH2O and incubated overnight at 25°C. After incubation, the water – now containing larval secretions – was removed by pipette, centrifuged, filtered (0.2 μm) and lyophilized. Freeze-dried material was resuspended in ddH2O at a final concentration of 40 mg/mL prior to use in antibacterial assays and characterization work.

The antibacterial assay employed was broadly based on the standard agar diffusion assay, whereby a colony of the target microorganism was picked off a stock plate and suspended in Ringer’s solution (10 mL). An aliquot of microorganism suspension (100 μL) was swabbed onto iso-sensitest agar plates (10 mL agar) containing pre-bored wells (4 mm). Secretory products (25 μL) were dispensed into the wells and the plates incubated at 35°C for 24 h. Radial zones of inhibition (mm) of bacterial growth around the sample wells were noted and used as an arbitrary measure of antibacterial activity.

The stability of sterile secretions and the assessment of molecular size of antibacterial activity were investigated using the clinically important strain epidemic MRSA type 16 (EMRSA 16) as target microorganism. To investigate the stability of larval secretions under a range of storage conditions, sterile secretions reconstituted from fresh freeze-dried stocks (40 mg/mL) were stored at −21°C, +4°C and +21°C. A freeze-dried preparation was also stored under the above conditions and reconstituted with ddH2O just prior to assay.

To provide a guideline for the molecular weight (kDa) of anti-MRSA activity, Amicon Centricrins (Millipore U.K. Ltd, Watford, Hertfordshire, U.K.) were employed with differing molecular weight cut-off (MWCO) filters. Crude secretion extracts (2 mL at a concentration of 20 mg/mL) were spun in Centricrins with a range of MWCO (3, 5, 10, 30 and 50 kDa) over a period of 2 h at high speed (2950 g). Samples of filtrate and retentate were taken at designated times through the spin (30, 60 and 120 min) in an attempt to optimize filtration. After each sample was taken during centrifugation, more ddH2O was added as a washing step to minimize the chances of filter blockage and ensure that maximal filtration was achieved. Samples of retentate and filtrate from each washing stage of the respective Centricon were collected, lyophilized and resuspended in sterile ddH2O to standard concentration prior to assay.

All data are expressed as an arithmetic mean, where n = 3 and error bars are a measure of the standard deviation.

Sterile larval secretions exhibited antibacterial activity against a range of bacteria. They were shown to inhibit the growth of Gram-positive bacteria *Staphylococcus aureus*, MRSA strains 15, 16 and *Streptococcus pyogenes* but exhibited limited inhibitory activity towards Gram-negative bacteria (Fig. 1). Growth of the Gram-negative *Pseudomonas aeruginosa* was slowed down when challenged with secretion on a solid media but no zone of complete inhibition was detected (as evidenced by halos/partial clearance zones). Secretions also exhibited haemolytic activity as indicated by the lysis of blood around the wells of crude extract on blood agar seeded with *Streptococcus pyogenes* and *Bacteroides sp*.

The inhibition of Gram-positive bacteria and the apparent inactivity towards Gram-negative bacteria is in agreement with the previously published studies, which have looked at the activity of larval secretions against bacterial liquid cultures (Thomas et al., 1999b). These liquid culture data, in combination with the solid media findings...
described here, demonstrate the antibacterial versatility of *L. sericata* secretory products under a range of media conditions (liquid vs. solid).

The larval extract proved to be highly robust, withstanding lyophilization, long-term storage, either as freeze-dried preparation or a reconstituted secretion (Figs 2A and B), in the fridge (4°C) and freezer (−21°C), and several freeze–thaw cycles. Lyophilization resulted in no detectable losses in subsequent antibacterial activity (unpublished data) but provided an important means of concentrating activity and standardizing secretions between naturally variable batches of larvae. After 16 weeks of storage either as a lyophilized powder or as a reconstituted secretion kept at either 4 or −21°C, only small losses in antibacterial activity against MRSA was observed. Another measure of robustness was the ability of reconstituted secretions to withstand several freeze–thaw cycles. All these factors are highly important in terms of development of a product for medicinal purposes.

By contrast to previous work (Simmons, 1935; Thomas *et al.*, 1999a), it has been shown here that boiling the larval extract resulted in a complete loss in antibacterial activity towards MRSA. One possible explanation for the failure to detect antibacterial activity after heat treatment is the lower sensitivity of the agar diffusion assay when compared to liquid culture assay employed by previous workers. Indeed, time-course experiments in our laboratory monitoring optical density of a range of bacterial liquid cultures with and without larval secretion revealed activity against some bacteria previously undetected by the standard solid media agar diffusion assays, most notably activity against the clinically important VRE.

The use of Centricon filters to probe the molecular size(s) of anti-MRSA activity revealed the presence of very small active moieties capable of passing through even the smallest filter. Activity was detected in all filtrates, down to the smallest pore filter (MWCO 3 kDa). Anti-MRSA activity was also detected in the retentates of the 10 and 5-kDa MWCO filters, indicating the presence of at least one additional larger antibacterial agent, distinct from the <3-kDa activity(s). The presence of more than one ‘antibacterial’ is perhaps unsurprising when one considers the range of ‘challenges’ a maggot is exposed to both in its natural carrion-feeding environment and within a wound.

Preliminary investigations used the filtrate from the 3-kDa spin and passed it through a micropartition device (Millipore U.K. Ltd) fitted with a 1-kDa filter; this revealed the presence of anti-MRSA activity in the resultant filtrate, providing evidence for the presence of at least one active compound with a molecular weight <1 kDa. Further evidence supporting the presence of low molecular weight...
antibacterial compound(s) in the crude larval extracts comes from the detection of numerous low molecular weight bands on sodium dodecyl sulfate–electrophoresis using high density gels designed for the separation of peptides.

This size of molecule, though small, is not uncommon in the realm of insect antibacterial peptides. In the early 1980s the first insect antibacterial peptide was purified and characterized from the haemolymph of pupae of the ceriposa moth challenged with bacteria (Steiner et al., 1981). The 37-amino acid-long induced cecropin peptide was cationic (positive charge) in nature and active against multiple Gram-negative bacteria including E. coli and Pseudomonas aeruginosa. A large quantity of insect peptides subsequently isolated belonged to the defensins group. The first isolated examples of these were both from the dipteran species, Sarcophaga peregrina (Robineau-Desvoidy) (Matsuyama & Natori, 1988) and the black blowfly Phormia terraenovae (Robineau-Desvoidy) (Lambert et al., 1989). The defensins were primarily active against Gram-positive bacteria with limited Gram-negative potency and were less than 5 kDa in molecular size. Interestingly, these features are in common with our L. sericata larval extract findings here. Many of the insect antimicrobial peptides have been reported to be constitutively expressed, especially those from within secretory cells (Hancock & Diamond, 2000) and do not require induction. Work in our laboratory investigating the effect of bacterial challenge on larval secretion antibacterial activity noted no observable differences between challenged and non-challenged larvae indicative of a constitutive activity.

In conclusion, this preliminary investigation has shown that the secretions from L. sericata larva possess significant antibacterial activity against a range of Gram-positive microorganisms, including some clinically important strains of MRSA. The activity/activities in this secretion were considered to be of low molecular weight, highly stable and a systemic part of the larva. They possess several characteristics consistent with insect antibacterial peptides. All these features point to the possible presence of compounds within the larval secretions of L. sericata that could prove highly useful in the fight against MRSA and other nosocomial infections.

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References


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