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Title

The quality control of a medicinal larval (*Lucilia sericata*) debridement device based on released gelatinase activity.

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Running Head

Radial-diffusion enzymatic assay for larval activity.

Abstract

Lucilia sericata Meigen (Diptera: Calliphoridae) larvae are manufactured worldwide for the treatment of chronic wounds. Published research has confirmed that the primary clinical effect of the product, debridement (the degradation of non-viable wound tissue), is accomplished by a range of enzymes released by the larvae during feeding. The quality assessment of larval activity is currently achieved during production using meat-based assays, which monitor insect growth and/or the reduction in substrate mass. To support this, we have developed a complementary radial-diffusion enzymatic assay (RDEA) to produce a visual and measureable indication of the activity of larval alimentary products (LAP) collected under standardised conditions, against a gelatin substrate. Using basic laboratory equipment and reagents, the assay is rapid and suited to high-throughput. Assay reproducibility is high (SD 0.06 - 0.27, CV 0.75 - 4.31%) and the LAP collection procedure does not adversely affect larval survival (mortality < 2%). As a cost- and time-effective method, it is suited to academic or industrial use, supporting good manufacturing (or laboratory) practice (GMP and GLP) as a quality control (QC) assay.

Key words

Lucilia sericata	Quality Control (QC)	Debridement
Gelatinase	Wound management	Medical device

Abbreviations

ANOVA	Analysis of variance
BB-50	BioBag 50
CV	Coefficient of variation
GLP	Good laboratory practice
GMP	Good manufacturing practice
LAA	Larval activity assay
LAP	Larval alimentary products
LDT	Larval debridement therapy
MHRA	Medicines and Healthcare Products Regulatory Agency
P	Probability value
PBS	Phosphate-buffered saline
PVA	Poly-vinyl alcohol
QC	Quality control
RDEA	Radial-diffusion enzymatic assay
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOP	Standard operating procedure
TBS	Tris-buffered saline

Introduction

In the context of wound debridement, the larvae of the greenbottle blowfly *Lucilia sericata* Meigen (Diptera: Calliphoridae) are bred to form the active component of wound management devices. In the present article, we describe the development of a new quality control (QC) assay to monitor consistency in the production of the 'BioBag' (BioMonde, Europe), a Medicines and Healthcare Products Regulatory Agency-licensed device (Manufacture Specials, Human; MS 25595) (MHRA, 2015), which contains live larvae, sealed inside a porous pouch.

The efficacy of larval debridement therapy (LDT) against a variety of chronic wounds has been reported in clinical trials and anecdotal observations, particularly regarding the rate and extent of debridement in comparison with standard treatments (Mudge et al., 2014, Gottrup and Jørgensen, 2011) and a reduction in the risk of requiring major amputation (Sherman, 2009, Waniczek et al., 2013). It is acknowledged that debridement is enzymatically-driven, both in terms of the degradation of non-viable tissue and the associated bioburden, which lead to the apparent promotion of healing (Sherman, 2014, Pritchard et al., 2015). Contained larval debridement devices such as the BioBag, permit the flow of wound fluid toward the larvae, as their alimentary products are passed out into the wound environment, enabling the exertion of degradative effects without direct contact between the insects and patient being necessary (Grassberger and Fleischmann, 2002).

Currently, the assessment of larval activity by industrial and academic institutions is achieved using meat-based methodologies (Wilson et al., 2016), whereby a reduction in substrate mass (usually pork) and/or the growth of

larvae is monitored over time (Blystone and Hansen, 2014, Cickova et al., 2015). Although visually impactive, the use of hand-made ‘burgers’ of pork muscle to measure debridement efficacy (Blake et al., 2007) has been criticised as a model for wound study (Steenvoorde and Oskam, 2008). Such tissues may lack homogeneity; preparation and execution are cumbersome, with several days being required to complete the assay for a single device or batch of larvae. However, they are suited to behavioural or developmental investigations (Saunders and Bee, 1995) and so to complement this larval activity assay (LAA), we have developed a radial-diffusion enzymatic assay (RDEA) which permits the rapid throughput of multiple samples following the standardised collection of larval alimentary products (LAP) from the manufactured debridement devices. As feeding is enzymatically-driven, with solid matter being liquefied extra-corporeally by the action of LAP (Casu et al., 1996, Hobson, 1932b), it is therefore suitable to harvest this material from the live larvae, for use as a substitute for whole organism studies.

Preparing a solution of LAP is common practice in research relating to larval therapy, and the approach has enabled the characterisation of substances produced by *Lucilia sericata* to facilitate its feeding (i.e. lipase, DNAse, glycosidases, matrix metallopeptidases, trypsin and chymotrypsin). Recognising debridement as being accomplished by a suite of biochemically distinct enzymes (Telford et al., 2012, Chambers et al., 2003, Telford et al., 2010, Brown et al., 2012), the RDEA system described in this paper incorporates a relevant substrate in an agar base, against which to determine the total gelatinase activity of LAP. As a readily-available laboratory reagent, gelatin is a cost-effective model for collagen, the most abundant component of the dermal skin layer and a structural protein found in the extracellular matrix (Byron et al., 2013).

Drawing from microbial zonal (Abraham et al., 1941) and immunological (Mancini et al., 1965) methodologies, we developed an enzymatic variation of radial diffusion (Bowles et al., 1990) which produces a visual and measurable indication of activity within six hours. Supported by a standardised procedure for the collection of LAP from the smallest but most commonly used debridement device in the UK, the ‘BioBag-50’, the RDEA assay requires only basic laboratory equipment; the gel/liquid arrangement may be modified according to industrial or academic need, and pre-poured diffusion plates are suited to scaling-up, manufacture and shipment to other sites. Furthermore, the levels of standardisation, reproducibility and validation required for QC and laboratory accreditation purposes are attainable with these methods.

Materials and methods

Collection of larval alimentary products

Sterile 'BB-50' debridement devices were kindly supplied by BioMonde (Bridgend, Wales) as shipped to practitioners: as live, newly-hatched *Lucilia sericata* larvae heat-sealed within a monofilament polyester yarn packet (2.5 × 4 cm) containing a poly-vinyl alcohol (PVA) foam 'spacer' (1 cm^3) for structural support, and moistened with sterile saline. For the purposes of this study, control BioBags were also produced devoid of larvae (**Figure 1**).

Sterile phosphate-buffered saline (Sigma Aldrich; Suffolk, UK) was added to each delivery tube (2.5 mL) inside a Class II microbiological safety cabinet, and the caps were replaced but not tightened. Passive collection was undertaken in the dark for 24 hours, without any additional food media. Larvae were able to circulate freely within the BioBag and to remain moistened, without full immersion, which could lead to drowning. Upon completion, all BioBags were immersed in just-boiled hot water for 60 seconds and transferred to 80% ethanol for storage, in

accordance with standard entomological procedure (Amendt et al., 2007, Smith, 1989). Larvae were removed from the bags and hand-counted, and those which were dead prior to preservation (discoloured and distorted larvae) were recorded separately. Collection solutions were transferred to sterile 1.5 mL micro-centrifuge tubes and the total protein concentration and specific chymotryptic activity estimates of each BioBag were determined as described previously (Chambers et al., 2003), prior to storage at -20°C. Control devices (devoid of larvae) were treated in the same manner.

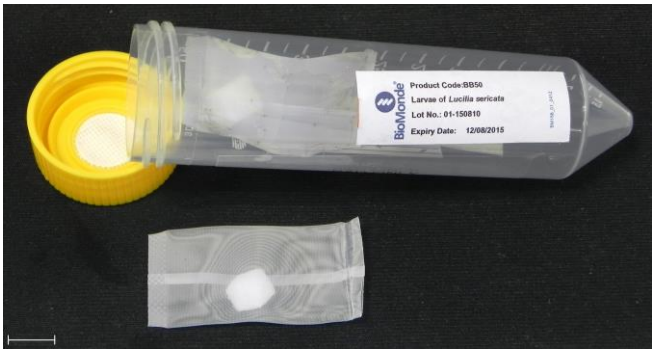

Fig. 1. Examples of the manufactured 'BioBag-50' debridement device; showing a BioBag containing larvae with a polyvinyl alcohol spacer inside the delivery tube, and a control BioBag devoid of larvae. Scale bar 10 mm.

Preparation of substrate base

Previous experimentation led to the formulation of a simple gel-base to incorporate the substrate, whilst providing the required tensile strength to permit the punching and removal of test wells, and to produce visible diffusion halos without overlap or distortion. The substrate base was prepared as 2% agar (Sigma Aldrich; Buchs, Switzerland) and 5 mg/mL porcine-skin gelatin (Sigma Aldrich; Steinheim, Germany) in tris-buffered saline (TBS, pH 8.2; 50 mM tris(hydroxylmethyl)methylamine, 150 mM sodium chloride) and microwave-heated until clear of particulates. Twenty millilitres of molten solution was transferred to 10 × 5.5 × 0.5 cm radial-diffusion plates (kindly supplied by The Binding Site; Birmingham, UK) using an automatic pipette filler. Gels were poured on a level surface and allowed to solidify before overnight refrigeration. Prior to use, 14 wells were punched using a 4 mm metal cork-borer and the contents removed using a sterile lancet.

Assay controls

As we were developing a new assay for larval enzyme activity, it was important to determine that all substrate degradation was derived from the insects alone. Therefore, a number of rigorous controls were incorporated and tested in the RDEA system.

Gelatin digestion: To confirm enzymatic action upon the substrate gel, a collagenase standard (10 μg/mL, TBS) from *Clostridium histolyticum* (Type VII; Sigma-Aldrich) was prepared as a positive control.

Plate substrate: To verify that LAP was acting upon the gelatin substrate, agar-only plates were produced and tested against LAP solutions with known enzymatic activity.

Plate buffer: To confirm the inactivity of the TBS used in the RDEA system, individual aliquots were prepared (as negative controls) for testing, from the stock solution.

Larval device: To confirm that the packaging surrounding the larvae was not contributing to the enzymatic action of LAP, each complete BioBag was supplied with a corresponding control device, comprised of delivery tube, containing a sealed polyester pouch with PVA spacer inside, but devoid of any larvae.

Larval buffer: To confirm the inactivity of the phosphate-buffered saline (PBS) used in LAP collection, individual aliquots were prepared for testing (as blanks), from the stock solution before and after transfer to the empty and active BioBags.

Larval food: To exclude the possibility that the measured gelatinase activity was derived from the sterile nutrient agar media produced by BioMonde (90 mm, commercially-controlled content) used for egg-hatching and initial larval feeding (< 24 hours), extracts were prepared by surface irrigation (1ml sterile PBS), gel punch (4x 10 mm discs created and macerated using sterile pipette tip) and gel punches (as previous) macerated in 500 µL sterile PBS. Protein concentration and chymotryptic assays were conducted as described previously. Constituent elution was confirmed using SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) analysis under reducing conditions, with 4-12% gradient gels (NuPage, Bis-Tris Midi 1.0 mm; Life Technologies, California, USA) prior to testing for potential background activity in the RDEA system.

RDEA analysis of larval alimentary products

Ten microlitres of collection solution from each individual BioBag was added to the upper row of each plate (wells 1 - 6), alongside controls as required (wells 7 and 8) of the same volume. Collection solutions from corresponding devices devoid of larvae were added to the lower row (wells 9 - 14), and all plates (with lids positioned, prepared in triplicate) were incubated at 37°C for 6 hours. Upon completion of the assay, lids were removed and the gels fixed within the plate, using glacial acetic acid, methanol and water (10:25:65), for 30 minutes, prior to staining with 0.05% Coomassie Brilliant Blue R-250 (Sigma Aldrich; Suffolk, UK) to confirm protein digestion. Halo diameters for gelatinase activity were measured (± 0.2 mm) using calibrated carbon-fibre digital callipers (Fisher Scientific; Pennsylvania, USA) as recommended in zone of inhibition studies (Hombach et al., 2013), on a transmitted light-box, and values were corrected to exclude the well diameter (4 mm).

Data analysis

Gelatinase activity is expressed as mean halo diameter (mm) with standard deviation (SD) and coefficient of variation values (CV %). Statistical analyses were performed using GraphPad Prism (v.6.05; California, USA). Data normality was confirmed using Shapiro-Wilk test; one-way analysis of variance (ANOVA) and Pearson's *r* correlation tests were used to compare the digestion activity of the BioBag batches and the relationship with larval number, respectively. A probability value (*P*) of < 0.05 was considered significant.

Results

RDEA controls

Gelatin digestion: Digestion activity was observed with all collagenase positive controls (**Figure 2**).

Plate substrate: No digestion activity was observed with any agar-only plates (not shown).

Plate buffer: No digestion activity was observed with any of the TBS negative controls (not shown).

Larval device: No digestion activity was observed with any of the control devices devoid of larvae (**Figure 2**).

Larval buffer: No digestion activity was observed with any of the PBS blanks (not shown).

Larval food: No digestion activity was observed with the larval media (**Figure 2**). No chymotryptic activity was associated with the larval media, protein concentration was estimated at approximately 3.5 mg/mL and constituents were resolved using SDS-PAGE (**Figure 3**), indicating the release of the potentially enzymatic food components.

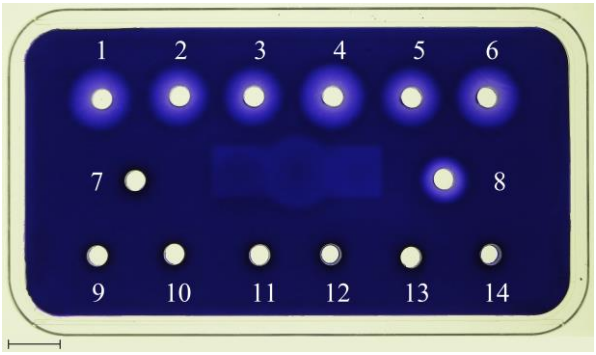

Fig. 2. Radial diffusion enzymatic assay (RDEA) for gelatinase activity of larval alimentary products (LAP) from six BioBag-50 debridement devices (wells 1–6) and corresponding devices devoid of larvae (wells 9–14) with larval food control [macerated in phosphate-buffered saline (well 7) and collagenase standard (well 8)], following incubation at 37∘C (6h) and Coomassie Brilliant Blue staining (0.05%). Darker 'edge artefacts' around negative wells demonstrate the lack of digestion in the presence of diffusion, post-staining. Scale bar: 10mm.

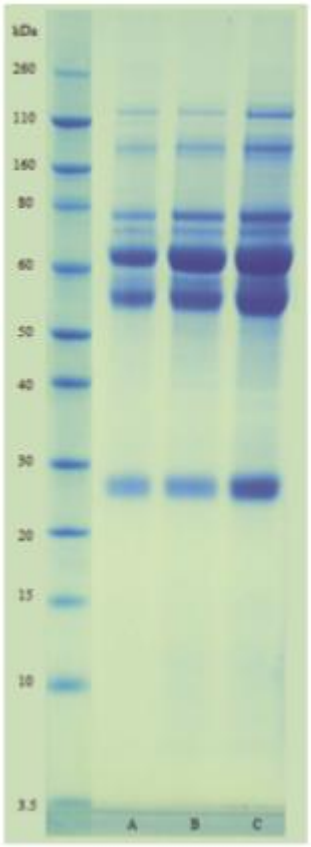

Fig. 3. Larval food plate constituents resolved using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (reducing conditions) using NuPAGE, 4–12% Bis-Tris Midi 1.0mm gradient gel with Novex Sharp Prestained Protein Standard (5µL, 260–3.5kDa). Samples(3µL)representextractionprocedures.ColumnA:surfaceirrigation [1mL phosphate-buffered saline (PBS)]. Column B: 4×10-mm gel punches macerated in 500µL PBS. Column C: 4×10-mm gel punches macerated.

Characterisation of collection solutions

Three separate batches of six BioBags with control devices were received over a 10-month period; characterisations of LAP collected under standardised conditions were undertaken for RDEA validation. Total protein content estimation and specific chymotryptic activity assays were conducted as internal benchmarks for the initial evaluation of LAP solutions and for confirmation of enzymatic action (**Table 1**). No values for protein concentration or chymotryptic activity were recorded for the control larval devices, TBS or PBS blanks (data not shown).

Table 1: Characterisation of larval alimentary products obtained from BB-50 debridement devices (n=6 per batch) across 3 batches, (SD).

	Batch 1	**Batch 2**	**Batch 3**
No. Larvae	109.3 (11.59)	94.33 (19.44)	153.5 (49.36)
Mortality (%)	1.347 (1.24)	0.548 (0.93)	3.918 (1.98)
Protein Concentration	26.27 (13.48)	22.75 (4.93)	52.46 (24.33)
Chymotryptic Activity	33.67 (18.07)	51.10 (5.28)	33.44 (9.45)

Protein concentration estimation (µg/mL), modified Bradford assay, in duplicate.
Specific chymotryptic activity (pmol AMC* released/min/µg protein), in duplicate.
AMC: Fluorescent 7-amino-4-methylcoumarin (AMC*) is released following cleavage of the substrate succinyl-alanyl-alanyl-prolyl-phenylalanyl-AMC) by chymotrypsin (Chambers *et al.*, 2003).

Digestion of gelatin by larval alimentary products

Digestion activity was observed in all instances with standard BioBags (Figure 2). Individual device replicates produced digestion halos with low standard deviation (0.06 - 0.27) and coefficient of variation (0.75 - 4.31%) values (**Figure 4**). One-way ANOVA revealed no statistically significant difference between the mean values of each batch (P= 0.0986).

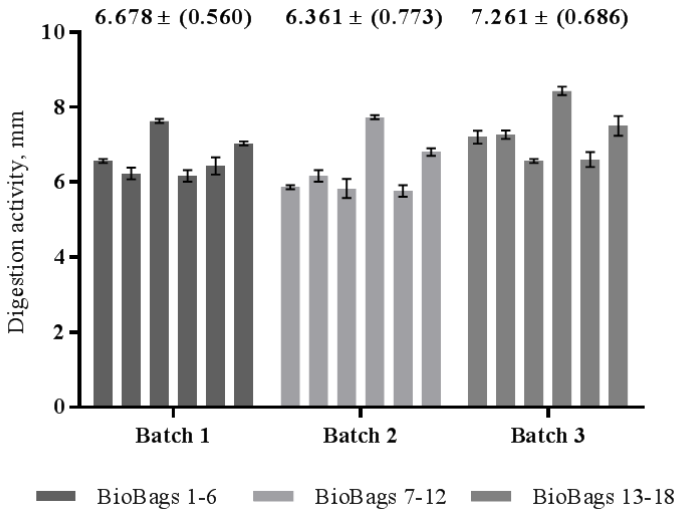

Fig. 4. Digestion activity (mm, in triplicate) of larval alimentary products against gelatin substrate (6h at 37∘C). The total dataset represents three separate batches of BioBag-50 debridement devices (n=6 per batch). Text in bold refers to mean±standard deviation intra-batch values.

Although consistent results for each BB-50 and corresponding replicates were achieved, a measurement range of 5.77 - 8.43 mm (± 0.2 mm) in the total dataset (n=18) was observed. Most notably, peaks in activity appeared to be associated with the largest number of larvae per BioBag within each batch (**Figure 5**). The potential relationship

between larval number and digestion activity was explored, showing the emergence of a significant correlation (**Figure 6**).

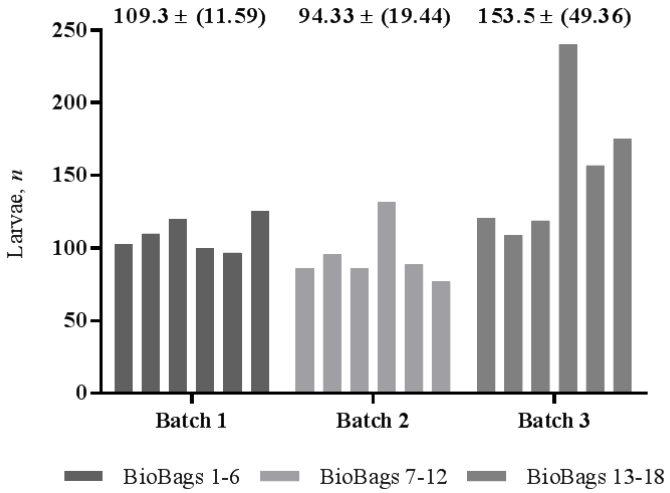

Fig. 5. Larva lnumbers per BioBag (hand-counted) for each batch. The total dataset represents three separate batches of BioBag-50 debridement devices (n=6 per batch). Text in bold refers to mean ± standard deviation intra-batch values.

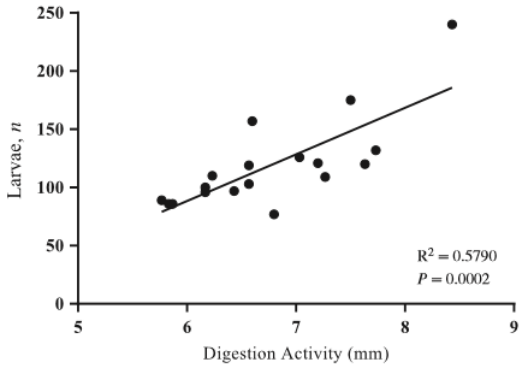

Fig. 6. Pearson's r correlation between digestion activity and number of larvae contained in each BioBag (n=18).

Discussion

The liquefaction and reduction of solid tissues as a result of larval feeding is evident in nature, and during laboratory rearing of sarcosaphrophagous blowflies, whereby animal tissues are usually provided to adults and their offspring (Hobson, 1931, Hobson, 1932a). The subsequent degradation is readily visible and generally rapid, and an assay based on the method of Blake *et al* (2007) is currently in use for the assessment of larval activity (Wilson et al., 2016)

A reduction in the mass of the substrate and/or the growth of larvae (length, weight, mortality) and progression to adult (pupariation, fly eclosion, subsequent oviposition etc.) are measured to determine the rate and quality of insect growth, to ultimately monitor colony fitness and output in mass-rearing establishments (Schneider, 2009, Singh and Moore, 1985). Suited to behavioural or developmental assessment, such as investigations relating to density-dependent feeding levels (Ireland and Turner, 2006) and larval dosage recommendations (Sherman, 2003) the approach does not lend itself well to standardisation, modification, rapid throughput or general handling in the laboratory.

The RDEA system incorporates a wound-relevant substrate and is suited to multiple sample analysis (up to 14 samples per plate); a clear advantage over meat-based methods (single samples, per meat assay). We have proven that all enzymatic activity observed was attributed to LAP action on the gelatin substrate, as shown by the lack of digestion with control devices devoid of larvae, agar-only substrate and background feeding biomaterials. Using only basic laboratory equipment and reagents, the assay is completed within 6 hours (37^{o}C), as opposed to 2 days (32^{o}C). With the added advantage of substrate and LAP homogeneity, reproducibility is high (SD 0.06 - 0.265, CV 0.75 - 4.31 %) and the standard operating procedure (SOP) for LAP collection does not adversely affect larval survival (mortality < 2%). As a cost- and time-effective assay, it is suited to academic or industrial use, and supports good manufacturing (or laboratory) practice (GMP and GLP).

In summary, we describe the formulation of a QC assay for use with manufactured larvae, to assist batch assessment in industry or for use in academic investigations, to lend further formality to an otherwise 'alternative' treatment, which although effective, we believe is under-utilised.

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