RESEARCH

Sterilization of *Chrysomya putoria* (Insecta: Diptera: Calliphoridae) Eggs for Use in Biotherapy

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ABSTRACT. Large-scale, quality-controlled laboratory production of fly larvae is needed for biotherapy. The objective of this study was to assess the action of glutaraldehyde on the sterilization of *Chrysomya putoria* eggs by applying pharmaceutical sterility tests. Egg masses with 0.600 g were divided into three parts of 0.200 g, the eggs were separated using sterile distilled water, and the suspensions obtained were mixed with activated 2% glutaraldehyde solution. After 15-min contact, the suspensions were filtered through Whatman filter paper, and the glutaraldehyde residue obtained in the filtrate was neutralized by rinsing with Tryptone Soy Broth. The treated eggs were placed aseptically on Petri dishes containing gauze moistened with sterile saline solution. About 10% of the sterilized mass was transferred to test tubes containing Tryptone Soy Broth and Fluid Thioglycollate Broth. The tubes were incubated, respectively, at 22.5 and 35.0°C for 14 d to verify egg mass sterility. The plates containing the rest of the eggs (90%) were sealed with plastic film and kept in a climatized chamber at 30°C/d, 28°C per night, 60 ± 10% relative humidity, and under a 12-h light period to assess insect viability and survival. Each experiment was carried out in triplicate using a biological class II safety cabinet. No change in color or turgidity was observed with the agent tested, proving the sterility of the product and that there was no trace of contamination. Forty larvae (in three replications) in the periods of 12, 24, and 48 h after sterilization, when transferred to diet, produced larvae, pupae, and total viability similar to the control (larvae without sterilization). However, for the 72-h treatment, larvae and total viability were significantly lower than for the other treatments. There was no significant difference for the pupal stage. The product tested was shown to be efficacious for use as a sterilizer of *C. putoria* eggs for all the parameters assessed.

Key Words: wound, glutaraldehyde treatment, screw worm, larval therapy, sterility test

Studies on necrobiontophage flies have triggered interest among entomologists and researchers because of the applicability to several areas of science, such as biotherapy (Sherman 2009), forensic entomology (Estrada et al. 2009, Ferraz et al. 2012), biological control by using some species as hosts for parasite microhymentoptera (Mello and Aguiar-Coelho 2009, Barbosa et al. 2010), and for their ability to produce occasional or accidental myiasis (Ferraz et al. 2010).

Biotherapy is a therapeutic technique that uses live, sterilized larvae of a particular fly species to debride quickly and precisely wounds with devitalized tissue. The larvae of necrobiontophage flies act on the wound by digesting dead tissue, cell remains, exudates, or necrosed wounds. The extracorporal digestion is due to the release of proteolytic enzymes (collagenases, trypsin, and chemiotrypsin), the mechanism by which the wounds are cleaned. Debridement is helped by the ingestion of liquefied tissue and the larvae dragging themselves over the wound bed, along with maceration by the mouth hooks of the insect (Sherman et al. 2000). Fibroblast migration is stimulated and helps to remodel the extracellular matrix with an increase in angiogenetic activity, antimicrobial activity that creates an antiinflammatory response, and proinflammatory inhibition that breaks the wound bed biofilm and alters its pH, so that the healing time is reduced (Prete 1997; Mumcuoglu et al. 2001; Horobin et al. 2006; Cazander et al. 2009, 2010; van der Plas et al. 2010; Zhang et al. 2010). Biotherapy has been used routinely in some countries of North and Central America and Europe as an efficient alternative to help heal wounds considered difficult to treat such as diabetic feet, vein ulcers, pressure ulcers, burns, and some types of benign tumors, abscesses, and osteomyelitis (Sánchez et al. 2004).

Biological film formation in infectious processes in tissues or associated with medical apparatus implants is a great therapeutic challenge. The extracellular matrix that involves the bacteria in the biofilms impairs the action of antibiotics, cells, and molecules of the host immune system (van der Plas et al. 2008). In vitro studies have shown that *Lucilia sericata* (Meigen, 1826) ES inhibits the formation or determines the disaggregation of biofilms formed by important pathogens such as *Staphylococcus epidermidis* and *Staphylococcus aureus* (Harris et al. 2007, Cazander et al. 2010). The disaggregation on the surface of the implant materials enhances the efficacy of antibiotics used during treatment (van der Plas et al. 2009, 2010).

C. putoria (Wiedemann, 1818) is a fly of the Calliphoridae family that has biological characteristics with the potential for use in biotherapy (Dallavecchia et al. 2010, Dallavecchia, D.L. 2013) because it presents necrobiontophage behavior and high reproductive capacity with treated egg mass production, essential characteristics for its use. This fly occurs abundantly in human environments (Gadelha et al. 2009), a fact that facilitates colony establishment and maintenance under laboratory conditions (Barbosa et al. 2004, Ferraz et al. 2010).

In the United States and Europe, the best known fly species for this purpose is *L. sericata*, a species that also belongs to the Calliphoridae family and is classified as a facultative parasite with fast development that favors in vitro rearing (Sherman 2003). However, the restricted geographic distribution of this species limits its use in larval therapy, and studies on other species with potential to substitute it are essential.

Every 30 s, a person has a limb amputated due to complications related to diabetes, a fact that causes numerous social and cultural problems and is directly related to the poor quality of life of these patients producing high government expenses with treatments, hospitalization, and subsidies, because the majority of these patients cannot continue working in addition to psychological problems that amputation generates not only in the amputate but also in their family (International Diabetics Federation 2005).

To be able to exploit the benefits of flies as therapeutic agents, sterile larvae must be produced to be applied to necrotic wounds, and fly egg sterilization techniques must be developed and fine tuned to prevent larvae from being vectors of unwanted pathogens to patients who have wounds that are difficult to heal (Baer 1935).

The objective of this study was to assess the action of glutaraldehyde as a sterilization technique on *C. putoria* eggs, a species of Calliphoridae native to Brazil with high potential for use in biotherapy.

Materials and Methods

The study was carried out in the Fly Study Laboratory and the Microbiology Laboratory in the Department of Microbiology and Parasitology of the Biomedical Institute of the Federal University of the State of Rio de Janeiro. Adult insects were captured using traps made by a polyvinyl chloride pipe following the model elaborated by Mello et al. (2007). Two hundred grams of chicken gizzard were inserted in the pipe as bait to attract insects. Traps were exposed for 12 h in locations of known fly infestation in the Fundação Jardim Zoológico in the city of Rio de Janeiro and then removed and taken to the Fly Study Laboratory. Captured insects were kept at $-10°$ C for 3 min until they stopped moving, so that they could be taxonomically identified under a stereoscopic microscope.

The insects were transferred to transparent 40- by 30- by 20-cm polyethylene cages with openings in the upper part covered by nylon fabric for aeration and on the sides to allow access to the inside of the cage. The adults were fed with a 50%-honey solution, water ad libitum, and chicken gizzard as a protein source and substrate for oviposition and larval rearing. Industrialized Superbom (Superbom Food Products Industry and Trade Ltda - Brazil (http://www.superbom.

com.br/produtos/mel)), honey was used, inspected, and approved by the Ministry of Agriculture (20 g = 60 kcal, 15 g carbohydrates). Larvae were reared following methodology by (Dallavecchia et al. 2010, Dallavecchia, D.L. 2013).

The egg masses were sterilized with 2% glutaraldehyde, a sterilizing liquid, used for hospital material. This agent acts quickly and efficaciously on Gram-positive and -negative bacteria, mycobacteria, fungi, and virus (ANVISA 2007).

A microbiological sterility test was used to assess the efficaciousness of the procedure by direct inoculation in culture medium using Tryptone Soy Broth (TSB) and Fluid Thioglycollate Medium as described in the 5th edition of the Brazilian pharmacopia (Brasil 2010).

Egg masses from ninth generation female *C. putoria* of the stock colony were transferred, using sterile tweezers, to a microscope glass slide and then weighed on semianalytical scales until 0.600 g total weight was gathered and divided into three equal parts of 0.200 g. The parts were then transferred to sterile Petri dishes and mixed with 4 ml of sterile distilled water to disaggregate eggs that were separated mechanically using a no. 0 paintbrush. The egg suspension obtained was transferred aseptically to a test tube containing a 20 ml of 2% glutaraldehyde solution and activated by raising the pH to 8.0–8.2 with the addition of 0.4% sodium bicarbonate and 0.05% monobasic sodium phosphate. After 15 min of contact, the tube contents were filtered through a 0.8-μm thick, 2-cm diameter Whatman filter paper disc placed in a sterile plastic base (100-ml Bactor Sterile monitor cup), supported by a Kitasato flask. Immediately after filtration, the filter paper was rinsed with 30 ml of TSB to neutralize any glutaraldehyde residue that might have toxic effects on the eggs. Under aseptic conditions, the eggs were transferred to a gauze surface moistened with 0.9% sterile saline

Fig. 1. Demonstrative scheme of the sterilization process sequence: (a) egg mass on chicken gizzard protein diet; (b) egg mass transferred aseptically to a Petri dish for weighing and separating for disassociation; (c) Bactor Sterile monitor filter cup and 0.8-μm Whatman filter paper with sterilized egg mass; (d) sample of sterilized egg mass transferred to test tube containing 10-ml TSB and FTB for the sterility test; (e) hatched larvae after 12 h transferred to chicken gizzard diet; and (f) larvae with normal development. Only the larva viability is shown in this study.

solution and placed on Petri dishes. At the end of the process, they were sealed with polyvinyl chloride plastic film and placed in an climatized chamber set at 30°C/d and 28°C per night, with 60 ± 10% relative humidity (RH) and a 12-h photoperiod (starting at 6:00 a.m.) to observe egg hatching and larval viability (Fig. 1).

At 12, 24, 48, and 72 h after sterilization, larval viability was assessed by randomly transferring, from each treatment, 40 *C. putoria* neonate larvae to 0.100 g of chicken gizzard placed in 100-ml beakers. These were placed in larger 500-ml beakers containing sterilized wood shavings that served as pupation substrate for mature larvae abandoning the diet. The beakers were sealed with nylon fabric and fastened with an elastic band. The same procedure was used for the control but with nonsterilized larvae. All treatments were placed in an climatized chamber (BOD) set at 30°C/d and 28°C per night, with 60 ± 10% RH and a 12-h light period starting at 6:00 a.m. After mature larvae abandoned the diet, the body mass was recorded for lots of five larvae on analytical scales (Geaka Ag-200-E, Gehaka Laboratório de Ensaio e Calibração Indústriae Comércio Eletro-Eletrônico Gehaka LTDA-Av. Duquesa de Goias, 235-Real Parque Morumbi-São Paulo-SP (http://www.gehaka.com.br/certificados-iso/) 0.1 mg precision) that were transferred to test tubes containing 2 g of sterile wood shavings. Daily observations were made until adult emergence.

For the sterility test, about 10% of the glutaraldehyde-treated egg mass was removed from the filter paper surface using a sterile brush and transferred to test tubes containing 10 ml of TSB and Fluid Thioglycolate Broth (FTB) that were incubated, respectively, at 22.5 and 35.0°C for 14 d. After the incubation period, the tubes were inspected visually for indices of bacterial growth, such as turbidity, film formation, or deposits. As there was no evidence at the end of the incubation period that indicated bacterial growth, the sample analyzed was considered sterile. All the experiments were carried out in triplicate, and the materials were handled in a biological class II safety cabinet (Fig. 2).

The following software was used to run analysis of variance of viability of larvae, pupae, and adults on time period treatments (control, T1, T2, and T3) and the significance ($\alpha = 0.05$) of differences in results submitted to Tukey's test, using a statistical software (BioStat 5.2 2010).

Results

The sterilization process by the 2% glutaraldehyde chemical agent did not impair egg hatching in the tested species. Regarding larval viability and development (Table 1), the larvae and total viability were significantly lower for the 72-h (T4) period compared with the other treatments (control, T1, T2, and T3). No significant differences were observed for the pupal stage among treatments. Although larvae and total viability were lower for the 72-h time period, they reached indices close to 70 and 50%, respectively, and were considered satisfactory. Transferring larvae within 12, 24, 48, and 72 h of life to diet allowed development of insects until pupal formation and emergence of adult forms without any evident sign of abnormality.

The microbiological sterility of the glutaraldehyde was proven through pharmacopeic sterility tests, thus proving the quality of the larvae and the reliability of the agent tested for use in this procedure.

Discussion

Several antimicrobial agents have been used to sterilize fly eggs for use in biotherapy (Table 2) of which the most used is sodium hypochlorite, alone or associated with boric acid (Dakin liquid).

Varzim (2005) tested eight sterilizing agents (formaldehyde, sodium hypochloride, potassium permanganate, chlorhexidin digluconate, Farmasept 500, Farmasept 800, Farmaseptplus—42.5% glutaraldehyde, and Ultrasept—4% glutaraldehyde) at different concentrations and times. To verify the effectiveness of the sterilization process, the treated larvae were transferred to test tubes containing peptonated water, homogenized, and then transferred to the surface of Plate Count Agar plates.

Table 1. Mean viability of the larva, pupa, and total stages of *C. putoria* reared on chicken gizzard diet in different treatments

Time (h)	Viability[b]		
	Larva (%)	Pupa (%)	Total stages (%)
Control	95.0[a]	98.0[a]	92.5[a]
T1 (12)	95.0[a]	95.0[a]	90.0[a]
T2 (24)	96.0[a]	90.0[a]	91.0[a]
T3 (48)	95.0[a]	80.0[a]	75.2[a]
T4 (72)	69.0[b]	66.0[a]	46.6[b]

T1, T2, T3, and T4: larva transferred to diet 12, 24, 48, and 72 h after hatching of sterilized eggs and control without sterilization.

[a]Forty larva per diet per three replications.

[b]Means followed by the same letter do not differ significantly by the analysis of variance, followed by the Tukey's test at 5% significance.
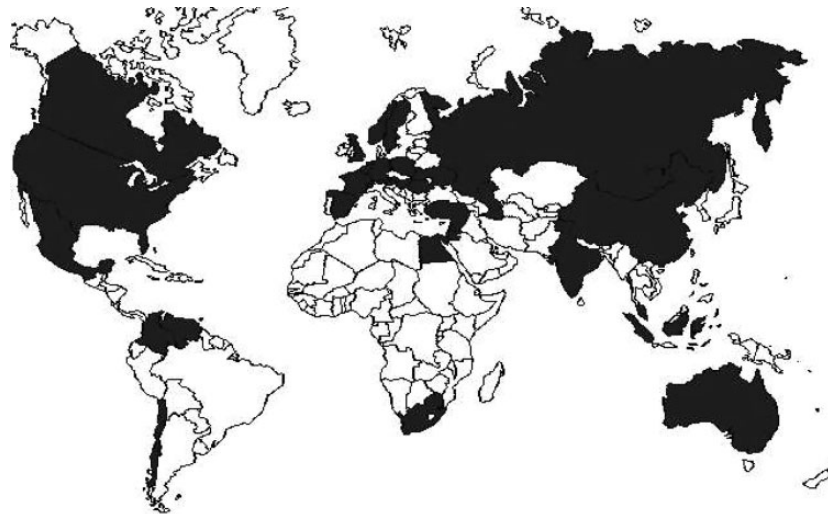

Fig. 2. Mapa Mundi illustrating the countries where biotherapy is currently used: the United States, Canada, Mexico, Venezuela, Colombia, Chile, Norway, the United Kingdom, Israel, Germany, Sweden, Austria, Hungary, Switzerland, Belgium, the Ukraine, Australia, Thailand, Turkey, China, Russia, France, Spain, Indonesia, Egypt, South Africa, India, Poland, Estonia, Slovakia, and Belgium (Dallavecchia et al. 2011).

Table 2. Sterilizers and their concentrations used for sterilizing the Calliphoridae egg surfaces

Family	Species	Sterilizing/concentration	References/country
Calliphoridae	*Phormia regina* (Meigen, 1826)	Mercuric chloride (1:1,000), ethyl alcohol 25%, and hydrochloric acid 0.5%	Baer (1931)/EUA
		Formal 10% and sodium chloride 0.85%	Fine and Alexander (1934)/EUA
	C. putoria (Wiedemann, 1818)	Sodium hypochlorite 0.5%, formaldehyde 1%, and farmaseptplus 1:4,000	Varzim (2005)/Brazil
	C. megacephala (Fabricius, 1794)	Glutaraldehyde 2% and Tween 20 5 ml	Dallavecchia et al. 2010, Dallavecchia, D.L. 2013/Brazil
	Lucilia caesar (Linnaeus, 1758)	Mercuric chloride (1:1,000), ethyl alcohol 25%, and hydrochloric acid 0.5%	Baer (1931)/EUA
	L. sericata (Meigen, 1826)	Formalin 5% and sodium hydroxide 1%	Simmons (1934)/EUA
		Sodium sulphite 2.5% and formaldehyde 2.5%	Mumcuoglu et al. (1999)/Israel
		Ortophtaldeído/(unspecified)	Contreras et al. (2005)/México
		Sodium hypochlorite 0.5% and formalin 10%	Figueroa et al. (2006)/Chile
		Acetic acid and streptomycin	Kocisova et al. (2006)/Eslovaquia
		Phenol 3% or sodium hypochlorite 0.5%	Sherman et al. (2007)/EUA
	Lucilia cuprina (Wiedemann, 1830)	Ultraviolet chlorhexidine and 70% alcohol	Mohd et al. (2005)/Malásia

Nitsche (2010) used 0.5 and 1.0% sodium hypochlorite solutions to sterilize *Chrysomya megacephala* and *C. putoria* eggs, using groups of 60 eggs, divided into three groups of 20 eggs for each species. Larval sterility was tested using a technique similar to that by Varzim (2005) in which, after sterilizing the eggs, Plate Count Agar plates and blood agar were used, but no contamination was reported in the samples.

In this study, it was attempted to sterilize large quantities of fly eggs to meet hospital requirements for use in biotherapy. Sherman et al. (2000) suggested that the idea would be to use 10 larvae/cm^2 of necrotic area. Generally, wounds considered small can receive the application of up to 200 larvae, whereas large wounds would receive the application of about 1,000 larvae for effective debridement. The professional should assess the extension of the wounds and the approximate number of larva that would be needed (Mumcuoglu et al. 1999, Jarvis 2000).

Preliminary sterilization tests of *C. putoria* eggs using 0.5 and 1% sodium hypochlorite were developed at different exposure times to sterilize 0.600-g egg masses, divided into three 0.200 g groups. However, while carrying out the sterility test according to the pharmacopeic protocol, the results were not satisfactory because contamination was observed in TSB and FTB that contained the sterilized eggs. The referred results served as a base to discontinue using sodium hypochlorite in this study and to research 2% glutaraldehyde as an alternative sterilizing substance (Dallavecchia et al. 2010, Dallavecchia, D.L. 2013).

Glutaraldehyde is an antimicrobial agent commonly used in disinfection in the form of 2% alkaline solution (pH 7.5–8.5). It acts against bacteria, fungi, and virus and is a sporicide in prolonged contact time. This action results from the alkalinization of sulfhydryl, hydroxyl, carboxyl, and amine groups that alter the nucleic acid and proteins of the microorganisms.

Sterile larvae are currently produced and commercialized by companies in the United Kingdom and Germany and laboratories in the United States, Israel, Sweden, Switzerland, Austria, and Ukraine (Mumcuoglu et al. 2001). In some countries, such as Brazil, there are few studies to date on biotherapy and larval sterilization even though it is a very old technique used in various countries. Biotherapy brings many benefits to wound treatment in diabetic patients and can contribute to indirectly minimize the damage caused by this disease. Its effects on wounds have already been proven by many studies (Mumcuoglu et al. 1999, Sherman et al. 2000, Contreras et al. 2005, Figueroa et al. 2006), and when compared with other debridement techniques, it is the most effective.

C. putoria and *L. sericata* are flies with larvae that develop very quickly. At temperatures from 27 to 30°C and 70 to 80% RH, reared on protein diets (natural and artificial), they take on average 1 d from egg laying to larval hatching, a mean of 5 (*C. putoria*) and 5.5 d (*L. sericata*) to develop to the third-stage larvae, and about 8 (*C. putoria*) and 7.5 d

(*L. sericata*) to reach the adult phase (Silva et al. 2008, Ferraz et al. 2012). Because they have biological and behavioral similarity with *L. sericata* and because they are abundant in the urban centers in some countries, especially in Brazil, *C. putoria* can be considered as a promising species for use in biotherapy.

The sterilizing agent, 2% glutaraldehyde, was shown to be effective in sterilizing larger egg mass volumes (0.600 g), and the toxicity attributed to the sterilizing agent in question, considered by Paulino (1999) as unviable due to high larval mortality rates, can be avoided if the egg masses are rinsed with a neutralizing agent during the sterilization procedure. The sterility test, following the ANVISA (2007) norms, is essential to ensure the product quality and the safety of patients.

The sterility tests carried out with TSB and Fluid Thioglycollate Medium detected groups of microorganisms that would not be detected by traditional plating with culture media methods used to date, such as anaerobic and filamentous fungi, when the appropriate temperature was used for their detection (22.5°C) in the TSB incubation; both the sterilization tests used to detect microorganisms were highly specific.

Studies and investments should be made and analyzed to increase larval production and make them available for use by the patients with wounds that are difficult to treat.

In this study, 2% glutaraldehyde was shown to be efficacious to sterilize the eggs and did not reduce hatching, larval, pupal, or total viability, or the normal cycle to the adult phase.

Acknowledgments

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