Identification of Antimicrobial Property of Rat-Tailed Maggots Surviving in Pig Excreta

Rajib Deb (✉ drajibdeb@gmail.com)

National Research Centre on Pig

Short Report

Keywords: Rat-tailed, maggots, piggery, Escherichia Coli, Salmonella typhimurium

Posted Date: July 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-669913/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

In the near future, antimicrobial resistance (AMR) will become a major danger to the global community. Despite the fact that India has many synthetic antibiotics, their irrational use is generating possible AMR in livestock. Furthermore, the expense of producing synthetic antibiotics is too expensive for the impoverished farming population. In this context, natural therapeutics are being sought. The current examination found that rat-tailed maggots from piggery excreta had antibacterial properties against *Escherichia coli* and *Salmonella typhimurium*, with minimum inhibitory concentrations (MIC) of 25–30 g/100 µl and 35–40 g/100 µl, respectively. Antimicrobial peptide sequences such as Dipteracin and Sarcotoxin were also found in the isolated hemolymph. In comparison to rice grained shaped maggot hemolymphs, comparative gene expression levels for both AMPs were substantially ($p < 0.05$) increased in rat-tailed maggots. The current study's findings may suggest that using natural maggots in animal husbandry waste as a therapeutic alternative decreases the unjustified usage of antibiotics.

1. Introduction

The “miraculous medicinal maggot” have shown a massive growth in literature in the past few years, with a huge number of reports on the topic of “antimicrobial agent”, “antibiofilm substrates”, “anti-inflammatory properties”, and “wound healing activities” cited on the web of science (Li et al., 2013; Naik and Harding, 2017). Maggots are thus proven to have promising therapeutics application with minimal adverse effects as well as cost effectiveness in comparison to the conventional therapy. Although it is increasingly recognized to utilize maggots in clinical applications, however scientific laboratory evidences are not enough to underlying the mechanism on the action of maggots (Yan et al., 2018).

The cost of synthetic antibiotic production would be very high and there are rising concerns on their biodegradability, causing accumulation in the environment and thereby eliciting the threat to the ecological balance (Kraemer et al., 2019). Moreover, antimicrobial resistance due to misuses of synthetic antibiotics in food animals is a burning issue in today's science (Mutu et al., 2020). In these current situation, searching of alternatives of synthetic antibiotics like natural maggots may be blissful.

The goal of the current platform was to find out if maggots living in piggery (*Sus scrofa*) excreta have antibacterial properties. Livestock excreta are one of the richest source of organic waste and the volatile substances exist in those organic waste are the main attracting factors by most of the fly species (Kim et al., 2021). Thus, attracted flies lays eggs on these excreta and further, the eggs will hatch and attain the next developmental stage like larva, pupa and then it became adult. Once the egg hatches, the larvae come out and survive; these larvae in the excreta are also called as “maggots”. These Maggots survive mainly by feeding on the decaying excreta, a rich source of organic and nitrogenous rich substances (Sarwar, 2020). Due to their antimicrobial inheritance properties, those maggots can happily survive in the filthy and microbial rich environment. Antimicrobial peptides (AMPs) like LS-sarcotoxin of blowfly *Luciliasericata* showed excellent activity against *E. coli*, *E. cloacae*, *Klebsiella spp.*, *Salmonella enterica*, *Citrobacter freundii* and *Acinetobacter spp.*, although they had little activity against *Pseudomonas*

aeruginosa. Similarly, LS-stomoxyn, on the other hand, had good activity against *Pseudomonas aeruginosa*, *A. baumannii* and most of the Enterobacteriaceae, with the exception of *K. pneumonia* (Hirsch et al., 2019). In this platform, we identified the antimicrobial property of rat-tailed maggots surviving in piggery excreta against *Salmonella typhimurium* and *Escherichia coli*.

2. Materials And Methods

2.1. Sample collection, copro-culturing and identification fly maggots

Piggery excreta/waste materials were collected from the Institutional Pig farm located at ICAR-National Research Center on Pig, Guwahati, Assam. Coproculture mixture using collected piggery waste/excreta was prepared by the addition of sawdust followed by spreading the coproculture on rectangular trays and keeping them in an open shady area for the flies to oviposit. Different fly larvae and pupae were then collected for their identification and further downstream processing.

2.2. Collection of the hemolymphs

The maggots were then washed with distilled water containing 0.1% Tween, washed with tap water for 1 min, and finally rinsed with 70% ethanol two to three times. Moisture was removed by using paper towels. The maggots were then transferred to a glass dish containing a slightly moist and fat paper tissue on the bottom. The glass dish was kept on ice to anaesthetize the live maggots. To prepare for collecting the hemolymph plasma, a hole was punctured in a 0.5-ml tube using a 23-gauge needle. Hemolymph was then collected in a sterile Eppendorf tube and maintained at or below 4°C. 60µg of phenylthiourea (PTU) crystal was quickly added into the tube containing hemolymph. The phenylthiourea will try to inhibit phenyloxidase present in the hemolymph and thus prevent the melanization of hemolymph. The hemolymph was then quickly frozen for stored at -80°C.

2.3. Characterization of the hemolymph for antimicrobial sensitivity

Pure culture plate of different organisms (Gram + ve/-ve) was utilized to study hemolymphs' antimicrobial activity from different types of maggots surviving in piggery excreta. Sterile Mueller-Hinton agar (MHA) plate and/ or selective nutrient agar (NA) plate was used for studying the antibacterial activity of hemolymph isolated from different maggots. Used the swab with the test organism to streak on the MHA and/ or NA plate for a lawn of growth. After the streaking is complete, plates were allowed to dry for 5 minutes. Antibiotic discs containing the hemolymphs extracted from the maggots were placed on the surface of the agar using sterilized forceps along with the standard antibiotic discs. Gently the discs press onto the surface of the agar using flame sterilized forceps or inoculation loop. Inoculated plates were incubated for 24 hours at 37° C. After incubation, a metric ruler was used to measure the diameter of the zone of inhibition.

2.4. Determination of Minimum Inhibitory Concentrations (MICs) hemolymph

Two to three colonies of *Escherichia coli* and *Salmonella typhimurium* were inoculated into their respective medium and incubated at 37°C for 18 h in a shaking incubator and then further cultured over 3 h under the same conditions. A total of 90 µL of bacterial culture mixtures were then transferred to a 96-well plate. 10 µl of serially diluted hemolymph were then admixed to wells, incubated at 37°C for 18 h. Absorbances were recorded at 600 nm to determine Minimum Inhibitory Concentrations (MICs) of the hemolymph.

2.5. Determination of Post Antimicrobial Effect (PAE)

Post Antimicrobial Effect (PAE) was determined by the methodology described by Dominguez et al. (2001). Two to three colonies of the respective bacteria (*Escherichia coli* and *Salmonella typhimurium*) were suspended in prewarmed Mueller–Hinton broth and incubated overnight at 37°C, diluted 1/2500 in the same prewarmed medium followed by incubation in at 37°C with agitation @ 50 rpm. The absorbance of the culture was recorded with a spectrophotometer at the wavelength of 660 nm till an absorbance of 0.1 was reached. An equal volume of the control culture, as well as the hemolymph, treated cultures were prepared separately. Kinetics on the antimicrobial effect of hemolymph on bacterial culture was studied for 1 hour and during that period, samples were withdrawn at every 10 minutes interval for the determination of bacterial concentration. The antimicrobial activity was halted by placing a 10⁻³ dilution of the bacterial suspension in the prewarmed Mueller– Hinton broth.

2.6. Identification of antimicrobial peptide (AMP) coding genes from the hemolymph

Hemolymph samples collected from the rat-tailed maggots were subjected for total RNA isolation followed by cDNA synthesis using protoscript® first-strand cDNA synthesis kit, NEB, as per the methodologies described elsewhere. A set of primers were designed to amplify two essential antimicrobial peptide coding genes viz—Diptericin and Sarcotoxin from the synthesized cDNAs from the rat-tailed maggot hemolymphs (Table 1). PCR reaction was performed in a 25µl reaction mixture having 1X PCR buffer (Sigma Aldrich, USA), 50 ng of cDNA, 0.5 µM of corresponding primers, 1.5 mM MgCl₂ (Sigma Aldrich, USA), 200 µM dNTPs (Sigma Aldrich, USA) and 1U Taq DNA Polymerase (Sigma Aldrich, USA). Amplified products were purified and subjected for sequencing.

Table 1
Designing Primers for AMP genes from the rat-tailed maggot hemolymph

Genes	Primers	Reference NCBI sequence
Diptericin	F: TCAGACATCAGACGTCAGCC R: CATCCGTATCCAGGTGCGAG	XM_034252399
Sarcotoxin	F: TCAATTACGTTGCAGAAGCA R: CGACTGTCCCAGGCTAATGG	XM_001955520
Drosophila Act5C	F: AAGGACTCGTACGTGGGTG R: ATCTTCTCACGGTTGGC	Lee et al., 2020

2.7. Real Time PCR based quantification of AMP genes among rice grained type and rat-tailed type maggots of piggery origin

cDNAs obtained from the hemolymph of rice grain shaped and rat-tailed maggots of piggery origin were assessed for the comparative gene expressions of Diptericin and Sarcotoxin AMP like genes using quantative Real Time PCR (Applied Biosystem). Gene expressions was normalized using the Drosophila melanogaster actin 5C (DmAct5C) gene. Primer sequences are illustrated in Table. Relative gene expression data using real-time quantitative PCR was analyzed by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

3. Results

3.1. Characterization of maggot hemolymph

Two different maggots of piggery waste origin were identified in the prepared copro-culture viz. rice grain shaped and rat-tailed maggots. The antimicrobial Sensitivity test (AST) revealed that rat-tailed maggot hemolymphs could develop a clear zone of inhibition (Fig. 1) against *Salmonella typhimurium* (14 mm length) and *Escherichia coli* (18 mm length), suggesting that evidence of their antimicrobial activities. We could not get any zone of inhibition against the rice grained shaped maggot hemolymphs.

3.2. Determination of the MICs of the hemolymphs

Antimicrobial activities of the rat-tailed hemolymphs were assessed by determining MICs against *Escherichia coli* and *Salmonella typhimurium*. The MIC range of hemolymph to *Escherichia coli* and *Salmonella typhimurium* was 25–30 µg /100 µl and 35–40 µg / 100 µl, respectively (Fig. 2).

3.3. Determination of Post Antimicrobial Effect (PAE) of hemolymph

To evaluate the *Post Antimicrobial Effect (PAE)* of the hemolymph, Gentamicin (@20mg/ml) and Streptomycin (@90µg/ml) were used against *Escherichia coli* and *Salmonella typhimurium*, respectively. Bacterial suspension without addition of hemolymph was treated as negative control for this experiment. Hemolymph with the concentration of 30 µg /100 µl and 40 µg /100 µl was used to evaluate the PAE on the bacterial culture of *Escherichia coli* (Fig. 3) and *Salmonella typhimurium* (Fig. 4), respectively. From the illustrated Figs. 3 and 4, it is clearly showed that the negative controls having an almost plateau OD readings in the range of 0.5 to 0.56.

On the contrary, positive controls where the respective bacterial culture was exposed to the selective antibiotic confirmed the bactericidal effect, where the OD readings declined from 0.5 to 0.2. The effect of rat-tailed maggot hemolymphs against the two bacterial species revealed their antibacterial activity. In both the cases (Figs. 3 and 4) OD value started with 0.5 and gradually declined during the period of 1 hour to approximately 0.3.

3.4. Amplification of AMP Genes from the rat-tailed maggot hemolymphs and its quantification

We amplified Dipteracin and Sarcotoxin like peptide sequences from the hemolymph of rat- tailed maggots and sequences were deposited in NCBI Bankit with the accession number MW836105 and MW836106, respectively. To quantify the gene expression levels for both the AMP related genes in the rat-tailed maggots having antimicrobial property, we used rice grain shapped maggots identified in pig coproculture which did not showed any antibacterial activity. Figure 4 clearly found that, expression level of both the genes were significantly ($p < 0.05$) higher in rat-tailed maggots in comparison to the rice grained shapped maggot hemolymphs.

4. Discussion

Despite the usages of synthetic antibiotics in human and animal practices, emergence of the Antimicrobial resistance (AMR) is predominating. AMR in coming days will be a global threat to the mankind. In order to overcome the potential AMR and also to achieve desired antimicrobial activity, development of safe as well as effective alternative to "synthetic antibiotic" is the need of the hour. In addition, the synthetic antibiotics are potential threat to the environment in causing the biomagnifications and accumulated in the human and animal cells. Moreover, they are not biodegradable and thus altering the ecological balance. In this condition cheap, cost effective and biodegradable potential natural antimicrobial agents are really going to be the next generation hope in order to overcome the existing AMR.

The problem of antibiotic resistance is becoming more and more serious. Insect antimicrobial peptide has been regarded as an important drug to solve antibiotic resistance. In this context, it is necessary to find a simple and easy way to obtain antimicrobial peptides. Recently the quest for the next generation of antibiotics is focused on natural peptides produced by animals or insects. Maggots are recognized as a "treasure house of antimicrobial peptides" as well as a good choice to produce and extract antimicrobial

peptides. Maggot has been hailed as the miraculous "medicinal maggot" for its diverse properties including antimicrobial, antibiofilm, anti-inflammatory, and wound healing activities. Current study clearly depicts the use of rat-tailed maggots surviving in the pig excreta as source of antimicrobial peptide in order to potentially exploit as an alternative to the existing synthetic antibiotics.

We identified the antimicrobial property of rat-tailed maggots survived in piggery excreta against two enterobacteria family *Escherichia coli* and *Salmonella typhimurium*. Colibacillosis is one of the important diseases of swine industry especially in neonatal mortality caused by pathogenic strain of *Escherichia coli* (Lupi, 2017). Among different serovars of Salmonella affecting swine population, *Salmonella typhimurium* stands out due to its zoonotic potential in human salmonellosis¹ (EFSA and ECDC, 2015) and its prevalent distribution in pork production (Arguello et al., 2013).

We found that, rat-tailed maggot hemolymph with the concentration of 25–30 µg /100 µl and 35–40 µg / 100 µl, respectively are enough to kill *Escherichia coli* and *Salmonella typhimurium*, respectively. Our findings corroborated with earlier studies, identified the role of normal hemolymphs to kill different kinds of bacteria (Sahalan et al., 2006; Basseri et al., 2016; Hirsch et al., 2020; Lee et al., 2020).

To determine the Post Antimicrobial Effect (PAE) of hemolymph against *Escherichia coli* and *Salmonella typhimurium*, we have chosen Gentamicin and Streptomycin, respectively as positive controls. Sahalan et al., 2006 used gentamycin as positive control for PAE of hemolymph from larvae and pupae of local fly species, *Musca domestica* and *Chrysomya megacephala*, against *Escherichia coli*. Effect of Streptomycin on the growth of *Salmonella typhimurium* is well documented. Singh et al., 2015 identified that Streptomycin can induced stress response in *Salmonella enterica* Serovar *Typhimurium*. Antibacterial Sensitivity Testing (AST) of various *Salmonella enterica* serovar *typhimurium* isolates across 11 antimicrobial compounds revealed that, genotyping performance of the bacterial isolates were improved to 0.89 sensitivity and 0.97 specificity with Streptomycin as antimicrobial agents with minor error rates. Present study envisaged the antibacterial role of rat-tailed maggot hemolymph for both *Escherichia coli* and *Salmonella typhimurium*.

Keeping in view on the role of antimicrobial peptides (AMPs) existing in the hemolymph of maggots, we amplified two AMP genes (Diptericin and Sarcotoxin like peptide) from the isolated hemolymph of rat-tailed piggery waste maggots. Recently, Hirsch et al., 2020 identified number of AMPs from rat-tailed maggots of *Eristalis tenax* and tested their antibacterial activity against a broad spectrum of microbes, including multidrug-resistant Gram-negative bacteria. From their study, it was speculated that cecropin-like and a diptericin-like peptides having the strong antimicrobial activity against the pathogens, achieved a good therapeutic window. Two maggot derived AMPs like LS-sarcotoxin and LS-stomoxyn were profiled to assess their suitability for systemic application in mouse model, found that these AMPs are suitable as lead candidates for the development of novel antibiotics (Hirsch et al., 2019). Thomas et al. 1999 confirmed the antibacterial activity of secretions against a range of bacteria, including *Streptococcus A* and *B*, *Staphylococcus aureus*, *Pseudomonas* and even methicillin resistant *S. aureus* (MRSA) in a preliminary laboratory study. To study the relevance of AMP gene expression on the antimicrobial

property of the rat-tailed maggots, we compared the expression levels of both the AMP genes among the rat-tailed and rice-grain shaped maggot hemolymphs. Our study interestingly insight that, expression levels of AMP genes are significantly higher in the rat-tailed maggots having antibacterial activity in comparison to rice-grain shaped maggots.

Currently in India scanty of systematic reports are available as for as the AMPs related to the fly maggots surviving in pig excreta. Future research needs to be organized for therapeutic evaluation of both the AMPs for their application as natural antibiotics.

Declarations

Acknowledgments

The authors are thankful to the Director, ICAR-National Research Center on Pig, Guwahati, Assam, India for providing necessary facilities to carry out the present research. It is to also acknowledge that, an Indian patent entitled "Antimicrobial activity of piggery waste medicinal maggots" has been filed with application number 202011052348.

Ethical treatment of animals

Not applicable

Declarations

Funding

This work was supported by the ongoing project entitled "All India Network Programme on Neonatal Mortality in Farm Animals" (Project Code: AINP-NM) funded by Indian Council of Agricultural Research, New Delhi

Conflict of interest

The authors declare that there are no conflict of interest associated with this study

Code availability

Not applicable

Authors contribution

RD generated the concept, laboratory works , manuscript drafting

Ethical Approval

This manuscript does not contain any experiments with human participants and/or animals performed by any of the authors

References

1. Arguello H, Sørensen G, Carvajal A et al (2013) Prevalence, serotypes and resistance patterns of *Salmonella* in Danish pig production. *Res Vet Sci* 95:334–342. <https://doi.org/10.1016/j.rvsc.2013.04.001>
2. Basseri HR, Dadi-Khoeni A, Bakhtiari R et al (2016) Isolation and purification of an antibacterial protein from immune induced haemolymph of American cockroach, *Periplaneta americana*. *J Arthropod Borne Dis* 10:519. <http://jad.tums.ac.ir/>
3. Authority EF (2016) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSa Journal* 15(12). <https://doi.org/10.2903/j.efsa.2017.5077>
4. Hirsch R, Wiesner J, Bauer et al (2020) Antimicrobial peptides from rat-tailed maggots of the drone fly *Eristalis tenax* show potent activity against multidrug-resistant gram-negative bacteria. *Microorganisms* 8:626. <https://doi.org/10.3390/microorganisms8050626>
5. Kim CH, Ryu J, Lee J et al (2021) Use of Black Soldier Fly Larvae for Food Waste Treatment and Energy Production in Asian Countries. *A Review Processes* 9:161. <https://doi.org/10.3390/pr9010161>
6. Kraemer SA, Ramachandran A, Perron GG (2019) Antibiotic pollution in the environment: from microbial ecology to public policy. *Microorganisms* 7:180. doi 10.3390/microorganisms7060180.
7. Lee KS, Yun EY, Goo TW et al (2020) Antimicrobial activity of an extract of *Hermetia illucens* larvae immunized with *Lactobacillus casei* against *Salmonella* species. *Insects* 11:704. <https://doi.org/10.3390/insects11100704>
8. Li X, Liu N, Xia X et al (2013) The effects of maggot secretions on the inflammatory cytokines in serum of traumatic rats. *Afr J Tradit Complement Altern Med* 10:151–154. doi 10.4314/ajtcam.v10i4.24.
9. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408. doi 10.1006/meth.2001.1262
10. Luppi A (2017) Swine enteric colibacillosis: diagnosis, therapy and antimicrobial resistance. *Porc Health Manag* 3:16. <https://doi.org/10.1186/s40813-017-0063-4>
11. Mutua F, Sharma G, Grace D et al (2020) A review of animal health and drug use practices in India, and their possible link to antimicrobial resistance. *Antimicrob Resist Infect Control* 9:1–13. <https://doi.org/10.1186/s13756-020-00760-3>
12. Naik G, Harding KG (2017) Maggot debridement therapy: the current perspectives. *Chronic wound care manag res* 4:121–128. <https://doi.org/10.2147/CWCMR.S117271>

13. Sarwar M (2020) Typical flies: Natural history, lifestyle and diversity of Diptera. In Life Cycle and Development of Diptera. IntechOpen. doi: 10.5772/intechopen.91391
14. Sahalan AZ, Omar B, Mohamed AY et al (2006) Antibacterial activity of extracted hemolymph from larvae and pupae of local fly species, *Musca domestica* and *Chrysomya megacephala*. Jurnal Sains Kesihatan Malaysia 4:1–11. <https://core.ac.uk/download/pdf/11490138.pdf>
15. Singh AK, Drolia R, Bai X et al (2015) Streptomycin Induced Stress Response in *Salmonella enterica* Serovar Typhimurium Shows Distinct Colony Scatter Signature. PLoS ONE 10:e0135035. <https://doi:10.1371/journal.pone.0135035>
16. Yan L, Chu J, Li M et al (2018) Pharmacological properties of the medical maggot: a novel therapy overview. Evid-Based Complementary Alterne, 2018. <https://doi.org/10.1155/2018/4934890>

Figures

Figure 1

Determination of Minimum Inhibitory Concentration (MIC) of rat-tailed maggot hemolymph on the *Escherichia Coli* and *Salmonella typhimurium*. It was identified that, 25-30 µg /100 µl and 35-40 µg / 100 µl of the hemolymph can lower the optical density (OD) from 0.5 to lesser than 0.4 in the overnight grown broth culture of *Escherichia Coli* and *Salmonella typhimurium*, respectively.

Figure 2

Kinetics on the effect of rat tailed maggot hemolymph (@ 30 μg /ml) against *Escherichia Coli* . Positive control represents Gentamicin (@20mg/ml)

Figure 3

Kinetics on the effect of rat tailed maggot hemolymph (@40 μg /ml) against *Salmonella tyhimurium* . Positive control represents Streptomycin

Figure 4

Relative mRNA expression of two AMP genes (Dipteracin and Sarcotoxin) among two different types of maggot hemolymph identified from piggery origin. RGM: Rice-grain shaped maggots. RTM: Rat-tailed maggots. a and b differ significantly at $p < 0.05$