Silkworm as an animal infection model for the screening of environmental, clinical and veterinary pathogens

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Abstract

Silkworm, \textit{Bombyx mori}, has passive immunity and can be infected by pathogenic bacteria. Therefore, it can be used as a robust bacterial infection model for screening of pathogenic isolates from various sources. In this work, 11 environmental, clinical and veterinary isolates were screened for pathogenicity using silkworm larvae by injecting bacterial suspension through their dorsal surface and observing response. Experimental conditions were established by using \textit{Bacillus thuringiensis SW_R_F_1}, \textit{Escherichia coli} O157:H7, \textit{E. coli} DH5a and 0.6% saline. Nine out of 11 isolates were detected pathogenic after screening. The biochemical and genomic analysis of the nine test isolates confirmed their pathogenicity. The LD\textsubscript{50} of \textit{Pseudomonas aeruginosa} 47D and \textit{Salmonella Typhimurium} 77 were $4.63 \times 10^7$ at 12 hours was $8.02 \times 10^7$ cells/100μl/gram at 24 hours respectively. These results indicated that silkworm exhibits differential pathological response for pathogenic and nonpathogenic bacteria, and can be used as an alternative to animal model for screening diverse isolates.

Key words: Silkworm larvae, animal model, pathogenic bacteria, non-pathogen, pathogen screening

Introduction

Animal model for toxicology testing to drug discovery,\textsuperscript{1,2} is a prominent issue all over the world for various reasons, offers a biological system in which researchers can test their hypothesis in vivo and can investigate phenomenon under study.\textsuperscript{2} Animal model can also be employed for microbial pathogenicity testing. With the recent advent of isolation of novel microorganisms,\textsuperscript{3} it is essential to challenge their ability of infect within biological system to understand host-pathogen interactions.\textsuperscript{4} Although use of mammalian models in bacterial infection study is well established, it is often a difficult job when a large number of bacterial strains need to be tested because this requires a large number of animals to be challenged. It seems costly, time consuming, requires complex experimental procedure and set-up as well as moral conflict from animal rights point of view. Silkworm, \textit{Bombyx mori}, an invertebrate animal excels those points and can be used as an animal model to screen pathogenic bacteria from various sources. Although being arthropod, insects have robust innate immunity and they show pathological response during infection.\textsuperscript{5} Along with short response time, another feasible feature of fifth instars silkworm larvae (SL) are large enough for ease handling in comparison to other model insects like \textit{Caenorhabditis elegans} and \textit{Drosophila melanogaster}.\textsuperscript{6} Silkworm does not require costly setting for rearing and easy to discard after experiments through simple autoclaving. Understanding of host-microbe’s interaction and screening of microbial isolates from different sources by silkworm animal model may provide an economic tool for developing countries like Bangladesh. Using the model to survey the pathogenicity of isolated bacteria from different sources will give valuable pieces of information to understand the dynamics between microorganisms in different niches of host or environmental source and their pathogenic properties. In the present work, SL been used to establish as a robust animal model to screen pathogenic bacteria from environmental, clinical and veterinary sources.
Materials and Methods

Bacterial Strains: Eleven bacterial strains isolated in our lab from clinical, veterinary seven and environmental samples (Table I) were used in this study. Additionally, E. coli O157:H7 and E. coli DH5α were used as controls. A B. thuringiensis SW_R_F-_1 strain that was isolated from flacherie diseased dead SL (our unpublished data) was also used for the present investigation.

Table I: Groupings of the different bacterial isolates and strains used in the study based on their pathogenicity screening test with their controls.

<table>
<thead>
<tr>
<th>Source</th>
<th>Organism</th>
<th>NCBI Genbank ID</th>
<th>Pathogenicity Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Environmental Isolates (Soil Samples)</td>
<td><em>Pseudomonas aeruginosa</em> SN28</td>
<td>KM373324</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em> SN26</td>
<td>KM373319</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus aureus</em> SN7</td>
<td>KM373314</td>
<td>B</td>
</tr>
<tr>
<td>2) Veterinary Isolate (Poultry Samples)</td>
<td><em>Salmonella Enteritidis</em> 56</td>
<td>KF188421</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella Typhimurium</em> 77</td>
<td>KF188422</td>
<td>A</td>
</tr>
<tr>
<td>3) Clinical Isolates (from Diabetic Foot Infection)</td>
<td><em>Pseudomonas aeruginosa</em> 47D</td>
<td>KM025388</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas stutzeri</em> 54D</td>
<td>KM025368</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus cereus</em> 44D</td>
<td>KM025373</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td><em>Swab</em> 39/Diab/MH1</td>
<td>KM025389</td>
<td>B</td>
</tr>
<tr>
<td>4) Insect Pathogen</td>
<td><em>Staphylococcus aureus</em> 39/Diab/MH1</td>
<td>NA</td>
<td>A</td>
</tr>
<tr>
<td>5) Laboratory Strain</td>
<td>E. coli DH5α</td>
<td>NA</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>E. coli O157:H7</td>
<td>NA</td>
<td>A</td>
</tr>
</tbody>
</table>

*Group A, rapid death; group B, gradual death; group C, lagged death.

Silkworm Larvae: Batches of healthy SL (Bombyx mori) were collected from Bangladesh Sericulture Research and Training Institute (BSRTI), Rajshahi during their first day after routine feeding of fresh mulberry leaves (Morus alba) in fifth instar larval state. The leaves were cleaned in water and spliced in pieces. Three different races of silkworm were used, namely Mistrari, BSR-95/10 and 95/22p. Those larvae were kept in room temperature (25-28°C) and 2-3 round of feeding were provided before the commencement of further experiments.

Phenotypic and genotypic properties of bacterial strains: Hemolysin and protease tests were carried out to assess the pathogenicity of the isolates by red blood cell lysis and proteolysis respectively. 5% sheep blood agar was used for Hemolysin as described previously eight and protease test was done on skimmed milk agar plate nine for all 11 isolates. The isolates were initially purified on nutrient agar media and a single colony was stabbed in both medium for 24 hours at 37°C.

Eight pathogenicity specific common genes were selected to detect in different isolates (Table II). DNA of all gram negative isolates were extracted by boiled DNA extraction procedure whereas gram positive organism’s DNA was extracted by using bacterial DNA extraction kit (Jena Bioscience GmbH, Germany) following manufacturer’s instruction. PCR reactions were carried out using ProFlex™ 3×32-well PCR system (Applied Biosystems, USA) with G2 master-mix (GoTaq® G2 Hot Start Polymerase, Promega, USA). Reaction volume was 15μl in all cases. The PCR products were separated in 1% agarose gel electrophoresis and their molecular weights were detected by using 1kb and 100bp ladder (Bioneer, Korea).

Experimental protocol: Five different treatments, such as insect pathogen B. thuringiensis SW_R_F[_1] and human pathogen E. coli O157:H7; and 0.6% saline, non-pathogenic E. coli DH5α strain along with no treatment control, were used to establish experimental protocol. After injecting each of the treatments using 1 ml 27G ¼˝ syringes into the hemolymph through dorsal surface of SL, mortality of SL was observed for 67 hours without feeding. There were 15 insects in each treatment groups. Dead SLs were detected by observing lack of their responsive movement. The data were plotted in a survival curve. Survival curve of different treatments were compared which illustrates the death pattern of SL that can be related with treatment injected in each group of larvae. Eleven environmental, veterinary and clinical isolates (Table I) were tested following above described method to validate the implication of SL model for the determination of pathogenicity of unknown isolates. Each treatment was done in triplicate, each comprising five larvae. A high dose of bacteria (∼ 10^9 cells per gram of larvae) was used.
Table II: List of pathogenic gene specific primers

<table>
<thead>
<tr>
<th>Pathogenic Gene</th>
<th>Detail</th>
<th>Primer Name</th>
<th>Sequence (5' – 3')</th>
<th>Amplicon Size (bp)</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hblA (Das et al., 2009)</td>
<td>Hemolysin BL</td>
<td>HblA1</td>
<td>GCTAATGTAGTTTCACCTGACATGAAC</td>
<td>834</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HblA2</td>
<td>AATCATGCCACTGCTGAGACATATAA</td>
<td>361</td>
<td>52</td>
</tr>
<tr>
<td>hlyA (Wang et al., 1997)</td>
<td>O Antigen Group 1</td>
<td>O157-4</td>
<td>AAGCTCCGTGTCGCTGAA</td>
<td>614</td>
<td>60</td>
</tr>
<tr>
<td>stx1 (Rasheed, 2014)</td>
<td>Shiga toxin gene</td>
<td>Stx1-F</td>
<td>ACCTGAGATATCTGATTGG</td>
<td>88</td>
<td>55</td>
</tr>
<tr>
<td>ssaT (Deekshit, 2013)</td>
<td>Secretion system apparatus</td>
<td>SsaT-R</td>
<td>ATGCCAATAATGGTGAT</td>
<td>780</td>
<td>55</td>
</tr>
<tr>
<td>cna (Switalski, 1993)</td>
<td>Collagen adhesion encoding gene</td>
<td>Cna-F</td>
<td>TCAGGCCGCGTTACAGAGACG</td>
<td>744</td>
<td>55</td>
</tr>
<tr>
<td>hlg (Switalski, 1993)</td>
<td>Gamma hemolysin</td>
<td>Hlg-F</td>
<td>ATTTGCTGAGATAGGC</td>
<td>937</td>
<td>55</td>
</tr>
<tr>
<td>oprL (De Vos, 1997)</td>
<td>Outer membrane protein</td>
<td>OprL-F</td>
<td>TAGGGACAACGATGACATACC</td>
<td>504</td>
<td>57</td>
</tr>
<tr>
<td>toxA (De Vos, 1997)</td>
<td>Exotoxin A</td>
<td>ToxA-R</td>
<td>CTTTCAGCTCGGCGCGACG</td>
<td>396</td>
<td>68</td>
</tr>
</tbody>
</table>

The data was observed for 44 hours and plotted in a survival curve and each treatment was compared with control conditions.

Postmortem identification of pathogenic bacteria: After collecting dead caterpillars from different treatment groups, their bodies were cut into pieces with sterile scissors, resuscitated in alkaline peptone water followed by culture in selective media such as mannitol salt agar, bismuth sulfate agar and ceramide which are used for the isolation of Staphylococcus, Salmonella and Pseudomonas respectively. Antibiogram of both bacterial culture from original treatment and culture after bacterial isolation from dead larvae of different treatments was done. The bacterial culture equivalent to 0.5 McFarland solution was spread with cotton swab on Muller-Hinton agar. After 30 minutes of incubation in room temperature, different antibiotic disks (Table III) were placed on the solid medium and resistance pattern of all bacteria observed after 24-hour incubation at 37ºC.

Determination of LD<sub>50</sub>: Median lethal dose (LD<sub>50</sub>) was determined for P. aeruginosa 47D, a clinical isolate and Salmonella sp. 77, a veterinary isolate along with positive control E. coli O157:H7 and negative control E. coli DH5α. A series of dilution of test isolates (10<sup>8</sup>, 10<sup>7</sup>, 10<sup>6</sup> and 10<sup>5</sup> cells/dose) were prepared and injected into SL through dorsal surface into hemolymph along with control treatments of high doses (10<sup>8</sup> cells/dose).

Table III: The antibiogram pattern of original culture and culture isolated from dead SL

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Original Culture</th>
<th>Culture Isolated from Dead SL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa 47D</td>
<td>AM, C</td>
<td>AZM, C</td>
</tr>
<tr>
<td>Salmonella Typhimurium isolate 77</td>
<td>LEV, VA</td>
<td>IMI</td>
</tr>
<tr>
<td>Bacillus cereus 44D Swab</td>
<td>PB</td>
<td>IMI, CIP</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa 47D</td>
<td>C, CL,</td>
<td>AZM, C</td>
</tr>
<tr>
<td>Salmonella Heidelberg isolate 53</td>
<td>LEV, TE</td>
<td>IMI</td>
</tr>
<tr>
<td>Staphylococcus aureus 39Diab/MH</td>
<td>DO, AM</td>
<td>CIP, IMI</td>
</tr>
</tbody>
</table>

* Abbreviation of antibiotic is following: AM (Ampicillin), C (Chloramphenicol), AZM (Azithromycin), CIP (Ciprofloxacin), LEV (Levofloxacin), VA (Vancomycin), IMI (Imipenem), PB (Polymyxin B), CL (Cephalexin), AZM (Azithromycin), TE (Tetracycline), DO (Doxycycline)
By plotting percentage of dead larvae against a range of doses in defined time and fitting the data in 2-degree polynomial regression model, LD₅₀ was determined by calculating the dose which represents 50% reduction of live SL.¹⁵ The generalized polynomial equation of two degree fitted with the experimental data is:

\[ y = \beta_0 + \beta_1 x + \beta_2 x^2 + \varepsilon \quad \ldots \ldots \quad [1] \]

Here, \( \beta_0, \beta_1, \beta_2 \) are parametric estimation of \( x, x \) is independent variable (log(dose)), \( y \) (％ of dead SL) is dependent variable and \( \varepsilon \) is estimated error of the fit. The statistical analysis and LD₅₀ determination were carried out on R (version 3.0.1) using ggplot2 package.¹⁶,¹⁷

**Results**

_Silkworm larvae act well as animal model for pathogen detection:_ After plotting percentage of survival data of silkworm from control treatments showed distinct survival pattern. _B. thuringiensis_ SW_R_F_1 caused a sharp death and _E. coli_ O157:H7 caused a continuous death with time which is indicated by a decline in number of live silkworm. In contrast, no treatment, 0.6% saline condition and _E. coli_ DH5α caused an initially lagged reduction of live silkworm larvae number. By comparing survival curve of twelve challenged isolates with control treatment, their pathogenicity was deduced and categorized into three groups (Table I) – highly pathogenic (sharp decline), pathogenic (continuous decline) and non-pathogenic (lagged decline) (Fig. 1).

_Post-mortem identification of pathogens confirms infections:_ Growth of bacteria which were isolated from dead silkworm of treatment group A (Table I), observed on respective selective media for _P. aeruginosa_ 47D, _P. aeruginosa_ SN28, _S. aureus_ 39/Diab/MH1, _Salmonella_ Enteritidis 56, _Salmonella_ Typhimurium 77 and _Salmonella_ Heidelberg 53. The antibiogram pattern of original culture and culture isolated from dead silkworm larvae of different treatments was observed and the antibiogram profile was delineated (Table II). The antibiogram pattern of all of the bacteria isolated from dead silkworm larvae of different pathogenic treatment showed similarity with that of the original culture, except for _Staphylococcus aureus_ 39/Diab/MH1, in which ciprofloxacin was resistant in post-mortal isolate but sensitive in original culture.

_Phenotypic and genotypic properties of the pathogens corroborate the model:_ In blood agar plates, β hemolysin was observed for _Pseudomonas aeruginosa_ 47D, _Bacillus cereus_ 44D Swab2 and _Staphylococcus aureus_ 39/Diab/MH1. All _Salmonella_ spp. showed α hemolysin in blood agar plates. In protease test _Bacillus cereus_ 44D Swab2 produced a clear zone and _Pseudomonas aeruginosa_ 47D produced hazy yellow zone around it. PCR was done with pathogenicity associated gene for detection of pathogenicity. Outer membrane protein gene oprL (504 bp) and exotoxin A gene _toxA_ (396 bp) were positive in both _Pseudomonas aeruginosa_ 47D, a clinical isolate and _Pseudomonas aeruginosa_ SN28, an environmental isolate (Fig. 2(a)). Hemolysin A gene, _hlyA_ (361 bp) was detected in _E. coli_ O157:H7. Secretion system apparatus _toxA_ _ssA_ T, which is a pathogenicity island II specific gene for _Salmonella_, was screened positive in all _Salmonella_ strains at 780 base pair position (Fig. 2(b)).

**LD₅₀ determination:** For _P. aeruginosa_ 47D, change in percentage of dead silkworm larvae across a range of varying logarithm of doses were fitted by polynomial regression model for data points at 12, 18, 24 and 34 hours (Fig. 3.a). For _Salmonella_ sp. Isolate 77, this was done for data points at 24, 34, 40 and 46 hours (Fig. 3.b). For _P. aeruginosa_ 47D, the 12-hour model and for _Salmonella_ sp. 77 both 12 and 40-hour model were found to be statistically significant at 90% confidence intervals (Table IV).

**Table IV:** Summary statistics of significant polynomial model fitted in LD₅₀ plot. Here, \( \beta_0 \) is estimate of intercept of fitted regression curve; \( \beta_1, \beta_2, \beta_3 \) are parametric estimates of \( x, x^2 \) and \( x^3 \) respectively; and \( \varepsilon \) is the estimate of standard error in the model (equation 1).

<table>
<thead>
<tr>
<th>Dose-Death Regression Model</th>
<th>Dose-Death Regression Model</th>
<th>Estimates of Parameter</th>
<th>Residual S.E.</th>
<th>Adj R²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \beta_0 )</td>
<td>( \beta_1 )</td>
<td>( \beta_2 )</td>
<td>( \beta_3 )</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 47D (12 Hour)</td>
<td>30</td>
<td>60.5</td>
<td>27.05</td>
<td>2.91</td>
<td>0.99</td>
</tr>
<tr>
<td><em>Salmonella</em> sp. Isolate 77 (24 Hour)</td>
<td>27</td>
<td>69.81</td>
<td>-2.54</td>
<td>4.21</td>
<td>0.99</td>
</tr>
<tr>
<td><em>Salmonella</em> sp. Isolate 77 (40 Hour)</td>
<td>32</td>
<td>63.99</td>
<td>-2.27</td>
<td>4.28</td>
<td>0.99</td>
</tr>
</tbody>
</table>

From the fitted curve of those models, LD₅₀ determined by finding log(dose) value in x-axis which correspond 50% dead silkworm number in y-axis. Calculated LD₅₀ was 4.63×10⁷, 8.02×10⁷.
and $6.83 \times 10^7$ cells/100μl/gram respectively (Table V).

**Table V: Calculation of LD$_{50}$**

<table>
<thead>
<tr>
<th>Dose-Death Regression Model</th>
<th>Log(x) at Y=50%</th>
<th>LD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> 47D (12 Hour)</td>
<td>17.65</td>
<td>$4.63 \times 10^7$</td>
</tr>
<tr>
<td><em>Salmonella</em> Isolate 77 (24 Hour)</td>
<td>18.20</td>
<td>$8.02 \times 10^7$</td>
</tr>
<tr>
<td><em>Salmonella</em> Isolate 77 (40 Hour)</td>
<td>18.04</td>
<td>$6.83 \times 10^7$</td>
</tr>
</tbody>
</table>

Fig. 1: Establishment of experimental control. *Bacillus thuringiensis* SW_R_F_1 causing a steep death, *E. coli* O157:H7 causing a continuous death while *E. coli* DH5α, saline and no treatment condition caused lagged death.

Fig. 2: Molecular detection of pathogenicity associated genes; (a) showing oprL in 504 bp and toxA in 396 bp position, (b) showing O157 in 361 bp and ssAL in 780 bp

Fig. 3: LD$_{50}$ determination for *P. aeruginosa* 47D (a) and *Salmonella* sp. 77 (b). With increasing dose, percentage of dead SL increased. Four curves were fitted in polynomial regression model for each organism for four different time and statistically significant fitted curve was used to calculate LD$_{50}$.

**Discussion**

The target of this study was to develop a rapid protocol to screen a large number of bacterial isolates from different sources. Advent of new technology has instrumented scientists in a way that they confront a large number microorganism of new species or strains, many of which are previously uncharacterized or poorly characterized. So, it is often required to characterize their pathogenicity. Although mice are a well establish animal model to test pathogenicity, it would be a hassle to use it for screening large number of isolates such as handling many mice, cost, rearing space and consideration ethical issues concerning killing of mammals in large number. In contrast, silkworm does not have those constrains and therefore can be a robust solution for screening problem.

*E. coli* DH5α, *E. coli* O157:H7, *Bacillus thuringiensis* SW_R_F_1 and 0.6% saline were used to establish experimental control along with a group of silkworm larvae with no treatment.
conditions. Their survival curve indicates that B. thuringiensis SW_R_F_1 and E. coli O157:H7, which are pathogenic to silkworm and human respectively, while used as positive control in this work caused a continuous fall of live silkworm larvae number after challenge dose. Negative treatment condition showed a decline after an initial plateau period. This distinctive trend between positive and negative control set the ground on which inference can be drawn about the pathogenicity of test isolates. Previously other studies also established experimental condition using human pathogen S. aureus five or silkworm pathogen nuclear polyhedrons virus\(^1\)\(^8\) as positive control. A limitation of current work is that LD\(_{50}\) is remained undetermined for control treatment conditions. But the experimental condition established here remains feasible for screening experiments.

Eleven bacterial isolates from three different sources were screened in silkworm larvae insect model and their pathogenicity was inferred by comparing survival curve of each test treatment with control. Previously, silkworm was used to evaluate pathogenicity of bacteria attached to cedar pollen.\(^1\)\(^2\) In this experiment, silkworm has been used to screen out pathogenic bacterium from a wide variety of sources. The insect model described in this work clearly identifies Salmonella Typhimurium isolate 77, P. aeruginosa 47D, S. aureus 39/Diab/MH1, Bacillus cereus 44D Swab2, P. aeruginosa 47D and S. aureus 39/Diab/MH1 showed protease positive test and produced zone of lysis in sheep blood agar. It should be noted that these two properties had been recognized as biochemical properties of a pathogen five. To detect pathogenicity associated genes, in P. aeruginosa 47D and P. aeruginosa N28 isolate, outer membrane protein gene oprL and exotoxin A gene toxA were detected by PCR. Hemolysin A gene, hlyA was detected in E. coli O157:H7 which was positive control in this work. Secretion system apparatus protein gene, ssaT, which is a pathogenicity island II specific gene for Salmonella, found in all Salmonella strains used in this study (e.g. Salmonella sp. Enteritidis isolate 56, Salmonella Heidelberg isolate 53, S. aureus SN7, Enterobacter sp. 44D/MH1, as pathogens despite of their sources.

Also, test bacteria under study were isolated from dead larvae, detected by growing in selective media and their antibiotic pattern was compared with original culture. In all cases, except S. aureus 39/Diab/MH1, the antibiotic resistance pattern was identical. This confirms that in most treatment conditions death of silkworm was caused by the infection by injected bacterium. The difference in antibiogram pattern for S. aureus 39/Diab/MH1 before and after post-mortem samples might be due to contamination during handling or preparation or the inoculated bacterium might have gained its’ resistance gene after injection by horizontal gene transfer. Salmonella Typhimurium 77, a veterinary pathogen isolated from poultry and a clinical isolate P. aeruginosa 47D were selected for LD\(_{50}\) determination. The LD\(_{50}\) for P. aeruginosa 47D was 4.63×10\(^7\) cells per 100 cells/100 μl/gram in 12 hours and for Salmonella Typhimurium 77, it was 8.02×10\(^7\) cells/100μl/gram for 24 hours. For P. aeruginosa, similar LD\(_{50}\) (=10\(^6\) cells per dose in OEP vaccination and = 10\(^8\)-10\(^9\) cells in three component vaccination) was observed in immunized rat, but not in non-immunized rat (=10\(^3\)-10\(^4\) cells).\(^1\)\(^9\) The same scenario was observed in case of Salmonella Typhimurium 77.\(^2\)\(^0\) The higher LD\(_{50}\) of gram negative organism in insect model previously was correlated with 60-fold increase of expression of C-reactive protein (CRP).\(^2\)\(^1\)

The putative 10 pathogens (from group A and B in Table I) were also characterized as pathogens by the presence of four specific genes responsible for pathogenicity and two biochemical properties. These biochemical tests were used to identify novel pathogenic genes five-inch silkworm infection model. Among the test isolates, Bacillus cereus 44D Swab2, P. aeruginosa 47D and S. aureus 39/Diab/MH1 showed protease positive test and produced zone of lysis in sheep blood agar. It should be noted that these two properties had been recognized as biochemical properties of a pathogen five. To detect pathogenicity associated genes, in P. aeruginosa 47D and P. aeruginosa N28 isolate, outer membrane protein gene oprL and exotoxin A gene toxA were detected by PCR. Hemolysin A gene, hlyA was detected in E. coli O157:H7 which was positive control in this work. Secretion system apparatus protein gene, ssaT, which is a pathogenicity island II specific gene for Salmonella, found in all Salmonella strains used in this study (e.g. Salmonella sp. Enteritidis isolate 56, S. Typhimurium isolate 77, S. Heidelberg isolate 53). The biochemical properties and molecular characterization of pathogenic genes detected in 10 putative pathogens identified by silkworm larvae model corroborated the authenticity and effectiveness of the insect larvae model as a method of detection of pathogens isolated from various sources. Only a small set of pathogenic gene and phenotypic characteristics was investigated, so negative results in these experiment does not necessarily conclude to lack of virulence of the isolates. Therefore, the informality, sensitivity and specificity of this
model are yet to be determined, and further more scrutinized experiments are required. It could be concluded that the minimal experimental time-length requirement, cost effectiveness, less space required for large number pathogen handling and the robustness of the insect model potentially excursing the model as an effective alternative method to animal model for pathogenicity detection for bacteria of different sources. The findings revealed that (i) Silkworm, B. mori, larvae may be used as an effective and authentic model for pathogen detection; (ii) The method is cost-effective, less time consuming, needs less space and robust.

Conclusion: This work presents a study of silkworm with conclusion that this insect can be used as a feasible animal model to screen pathogens of wide range of origin simultaneously. Silkworm insect model can also help to classify the level of pathogenicity of screened pathogens. LD50 of different pathogen in this model can provide a quantitative way to compare this model with another higher animal.

Authorship Statement

Arafat Rahman has carried out all wet-lab experiments and data analysis. Md. Aftab Uddin contributed on SL rearing and supply. Munawar Sultana and M. Abdul Malek take part on study design result interpretation. M Anwar Hossain take part in developing hypothesis and concept, design of experiment and finalization of the manuscript. Everyone has taken part in this article writing process and approved the manuscript.

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References


