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IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF *VIBRIO CHOLERAE* FROM THE MIDGUT OF SILKWORM *BOMBYX MORI* (LINN.) LEPIDOPTERA; BOMBYCIDAE.

***PRADHAP. M. AND SELVISABHANAYAKAM**

Department of Zoology, Annamalai University, Annamalainagar – 608 002

PRADHAP. M
Department of Zoology, Annamalai University, Annamalainagar – 608 002

***Corresponding author**

ABSTRACT

Seven different species of bacteria were isolated from the midgut of infected Silkworm *Bombyx mori* in the 4th instar stage. Out of seven four strains were observed as lethal to host. These isolates were undergone for identification by physico-chemical characterization identified as Gram-negative, facultative anaerobic bacilli proteobacteria *Vibrio sp. Vibrio* strains are pathogenic or opportunistic pathogens. So this particular strain was termed to using Sodium Dodecyl Sulfate (SDS) polyacrylamide gel electrophoresis as per Laemmli method. The result had shown the presence of 61kDa (kilo Dalton) and 18kDa proteins in the protein band. Purification and characterization of protein profile revealed that the silkworm-killing activity was due to the presence of 61kDa and 18kDa protein, because of this the particular molecular weighted proteins are having the tendency to secret extracellular toxin and kill the host namely Knottins (small inhibitors, toxins, lectins) folded omega toxin like protein. Finally this particular species were undergone for 16s rRNA sequence to the clear understanding of phylogenetic relationship.

KEYWORD

Facultative, Opportunistic pathogen, SDS, Extracellular toxin, Phylogeny

INTRODUCTION

The Silkworm (*Bombyx mori*) is an easily bred invertebrate animal used for basic studies because of its importance in sericulture. Silkworm *Bombyx mori* is one of the best infection model, (Andrew *et al*., 2004) in which silkworms are killed by the microorganisms that are virulent in humans, such as *salmonella* and *Vibrio spp*. opportunistic pathogenic strains of Enterobacter and Vibriacea family. Generally invertebrate animals such as *Caenorhabditis elegans* (*C. elegans*) and *Drosophila melanogaster* have been used for the purpose of economic and ethical considerations and have contributed to our understanding of the molecular basis of host–pathogen interaction (Chikara kaito *et. al*., 2005). The Silkworm is large enough sample to understand the activities, comparably which is difficult in *C. elegans* and Drosophila larvae. Therefore, it is possible to quantitatively evaluate the virulence of various bacteria or true fungi and to identify their virulence genes in the silkworm infection model. In generally opportunistic bacterial pathogenic infections of hosts consists of the following stages; attachment to and colonization of host surfaces, invasion of the epithelial cell layer, and systemic dissemination of the microbes. Animal models are important for the identification and evaluation of bacterial strains that are required for virulence and to study infectious stages at a molecular level.

Insects are known to have both cellular and humoral immune systems which together form a potent defense against invading bacteria (Boman and Hultmark, 1987; Kimbrell 1991). In cellular immunity mechanisms, such as phagocytosis and encapsulation are operative (Boman and Hultmark, 1987; Dularay and Lackie 1985; Rizki and Rizki, 1984), while humoral responses mainly involve the production of a variety of antibacterial proteins that induced or increased in response to infection. The antibacterial protein identified in many insects operates cumulatively against a wide range of Gram-positive and Gram-negative bacteria. These antibacterial proteins can be categorized by homology as belonging to one of the several families, namely, the Checkreins (Boman and Hultmark, 1987; Dickenson *et al*., 1988; Hultmark, 1980; Samakovlis, *et al*., 1990, 1991), attains (casteels *et al*., 1990; Hulmark *et al*., 1980) and Diptericins (Dimarcq *et al*., 1990; Wicker *et al*., 1990). The Silkworm *Bombyx mori* an economically important insect is a host for different pathogenic microorganisms. Different geographically differentiated genotypes of Silkworms are known to show varying degrees of tolerance to different pathogens (Chitra *et al*., 1975). Although earlier worker have characterized ceresin-like antibacterial proteins (Morisshima *et al*., 1990) and lysozyme (Powning and Davidson, 1973), to what extent they are involved in the hum oral response in Silkworm remains to be understood (Abraham *et al*., 1994). In the present study we have examined the toxic nature of opportunistic pathogenic bacterial protein involved and leaded to the lethal situation in lifecycle of Silkworm *Bombyx mori* (Linn.) in 4th instars stage.

In recent years a strong trend in microbial systematic has emerged to define taxa for the phylogenetic analysis and use of direct molecular characterizations, e.g., DNA

base composition, DNA-DNA hybridization, gene sequencing, etc., Laboratory methods for identifying bacteria have relied heavily on the use of either simple or differential microbiological tests such as the Gram stain, the growth on common (or exotic) diverse substrates, and subsequently the specific use of biochemical tests. These methods have revolutionized studies in bacterial evolution but, initially at least, did comparatively little for the practitioner who simply needed to identify a particular organism in a given environment or specimen. Thus, in the face of important breakthroughs, the arduous processes of isolating pure cultures and conducting the traditional procedures for culture identification have continued largely unabated. Over the last 10 years, there has been a considerable expenditure of effort to develop methodologies for rapid identification of microorganisms by using a variety of novel procedures. One of the most promising of these has been the use of DNA probes that are specific for an organism's 16S rRNA. There are lots of advantages in using the 16S rRNA methodology, not the least of which is the huge data base of sequence information that is currently available. Almost 1,000 16S rRNA sequences have already been documented for bacterial phylogenetic studies, and this data base includes a large variety of bacteria, including pathogens.

From a technical perspective the 16S rRNA sequence segments can serve as molecular probes, which will allow for species identification of any bacterium. In other instances, 16S rRNA probe hybridizations can give a more general grouping identity (at the genus or family level), depending on the application required.

MATERIALS AND METHOD

Insect immunization and collection of sample:

Target organism: *Vibrio sp*. from the gut of Bombyx mori.

Silkworm Bombyx mori (Linn.) larvae were reared on fresh mulberry leaves under ambient conditions as per the standard rearing method (Krishnaswami, 1978). To study induction kinetics of opportunistic bacterial pathogen activity larvae were reared in a unhygienic condition and also provided with moisture mulberry leaves. Fortunately while reaching the fourth instar stage worms were infected sample were collected and analyzed.

SDS-PAGE Electrophoresis:

After electrophoresis, according to the Laemmli's technique, two protein stain procedure were tested (Coomassie blue and silver stain). Best results were obtained with 0.5% Triton X-100 for 4 h and the silver stain procedure of Oakley . Protein profiles analysis showed that the method is reproducible and distinguishes not only species but sometimes also subspecies.

The cells were collected from 50 ml of the culture medium by centrifugation by 7,600 rpm at 4° C, resuspended in 500 ml of double-distilled water, and solicited on ice with a tip syndicator five times for 10 s each. After fornication, protein extracted, the extracted protein samples were boiled with Laemmle buffer (Laemmli, 1970) [1% SDS, 5% Mercaptoethanol, 0.05% Bromophenol blue in 25 mM Tris-HCl, 10% glycerol, pH 6.8] for 10 min and electrophoresis on 12% SDS-PAGE. Protein electrophoreses profile in gel was stained with Commissar blue R-250 (0.2%) and the bands were scanned using CCD camera (Kodak Megaplus, Model 1.4; resolution 1,024 ´ 1,024 ´ 256 gray levels). Protein patterns were analyzed by using an image-processing apparatus (BioImage; Millipore) with an Escherichia coli DH5a pattern as the standard. The patterns were compared by using the

information on apparent molecular masses of the bands, band spacing, and band intensity and it was calculated the relative mobility of proteins from TCE with peptone grown cells, pattern based on the migration rate of the standard molecular weight marker.

Selection of primer:
All the 16S rDNA sequence data were obtained from the DDBJ, EMBL, and GenBank databases. The 16S rDNA data sequence was available. The 16S rDNA sequence of *Bombyx mori* was aligned with the personal computer software Clustal V (Higgins, et al., 1992.).

Culture of target organism: Smear taken from the gut of the diseased Bombyx mori was used in the study. The cultures were grown overnight in nutrient broth with constant shaking (150 rpm), at 37 °C.

DNA Isolation and PCR amplification:
Genomic DNA was extracted as described by using Axygen kit for PCR amplification. Genomic bands are clearly visible on the gel image. In the first well the marker DNA called λ DNA digested with Hind III was added. The concentration of DNA was quantified using the instrument called NanoDrop. It estimates the amount of DNA (in nanograms) present in 1µl spectrophotometrically. Oligonucleotide primers 27f, 1492r (Weisburg et al. 1991) which are derived from the conserved sequences of the bacterial 16S rRNA gene, were synthesized by KeboLab (Sweden). PCR was performed in a total volume of 50 ml containing 10-20ng of template DNA, primers (0.1mM), dNTPs (200mM), and 1 U Dynazyme DNA polymerase (Finnzymes Oy, Finland). PCR amplification was performed in a GeneAmp PCR system (Perkin Elmer) according to the following program: One milliliter of the cell culture was centrifuged at 13,000×g for 5 min and the pellet was washed twice with distilled water. The washed pellet was resuspended in 100 µl of 0.125% sodium dodecylsulfate and 0.05 M NaOH (1:1). The suspension was incubated at 95°C for 15 min and kept at −20°C until used. One micro liter of the cell legate was used for PCR amplification. The 20-µl reaction mixture consisted of 15 mol of each Bacterio-specific primer (27f:5′-AGAGTTTGATCMTGGCTCAG-3′, and the universal primer 1492r: 5′-TACGGYTACCTTGTTACGACTT-3′), 1.9mM MgCl2, 0.2mM each of dNTP, 0.5 U of DNA polymerase, 1× PCR buffer, 0.05% Tween 20 and a DNA template. Amplification was carried out in a thermo-cycler (GeneAmp PCR System 2400,) using a temperature programme consisting of initial denaturation (10 min at 92 °C), 30 cycles (denaturation for 45 s at 92 °C, annealing for 15 s at 50 °C, extension for 2min at 72 °C) and final extension for 2 min at 72 °C.

For PCR amplification of the almost complete 16S rRNA genes, the bacterio-specific and universal PCR primers (27f: 5′-AGAGTTTGATCMTGGCTCAG-3′, and 1492r: 5′-TACGGYTACCTTGTTACGACTT-3′) were used. The 20-µl reaction mixture consisted of 6 pmol of each primer, 1.6mM MgCl2, 0.2mM each of dNTP, 0.75 U of DNA polymerase, 1× PCR buffer, 0.05% Tween 20 and 1 µl of the cell lysate. Amplification was carried out using a temperature programme consisting of initial denaturation (10 min at 92 °C), 30 cycles (denaturation for 45 s at 92 °C, annealing for 15 s at 50 °C, extension for 2min at 72 °C). The amplification products were examined by electrophoresis in a 1% agarose gel and 1× Taq Pol buffer at 2.5 µl and documented with the Gel Doc 1000 Documentation System.

Sequence analysis
The isolated PCR products (genes for 16S rRNA) were purified with QIA quick Gel Extraction Kit (Qiagen, Germany) and sequenced at the Institute of Microbial

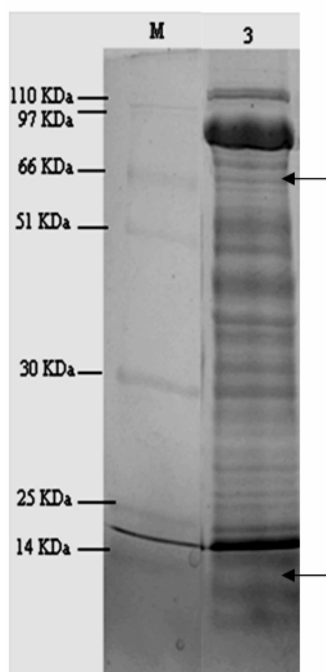
Technology, Chandigargh. The DNA was quantified with Nanodrop instrument. Standard protocol for cycle sequencing using ABI Prism 377 DNA Sequencer was used with following sequencing primers: 27f, 1492r, 1100r, 685r and 533f for each sample. All reference sequences were obtained from the GenBank database. The sequences were automatically aligned (multiple sequence alignment) by the Clustal W program (Higgins et al., 1992 and Thomson et al., 1994), using the default settings.

Nucleotide sequence accession numbers:
The nucleotide sequences of the 16S rRNA genes from the strains *Vibrio cholerae* were submitted to the GenBank nucleotide sequence database, under accession number GU086161.

RESULTS AND DISCUSSION

SDS-PHAGE

The purified protein elute after cytochrome on the gel fillteration column there are nearly 23 bands were obtained and the molecular weight of 61 kDa and 18 kDa are having the tendancy to produce serotoxin inside the cell. These particular molecular weighed proteins are characterized as serotoxin producing standard proteins. Exotoxin secreted into the cell and body fluid of the host organism that leads to cut down the calcium pathways (Barbara, 1995) and finally host becomes to scarify its life.



Design of the PCR primers.

In the present experiment, it has been observed that the design yielded 1428 base pairs. Altogether five different primers (27f, 1492r, 1100r, 685r and 533f) were tested in this assay. A set of two forward and three reverse primers were used. The forward primer 27f and the reverse primer 1492r showed coincidence with the *Vibrio cholerae*. Both the primers 27f and 1492r reacted with *Vibrio cholerae* at the annealing temperature of 50°C. The reactivity of the primer sets was reduced with increasing annealing temperature, but interspecies differentiations with these primers were

incomplete. The primers 1100r, 685r and 533f did not show any reaction with *Vibrio cholerae* even at the annealing temperature.

Universal PCR primers were already published by Lin, C.K. and Tsen, H.Y., 1996. Use of two 16S DNA targeted oligonucleotides as PCR primers for the specific detection of *Vibrio* in foods. Lin and Tsen (1996) for detection of *Vibrio* strains based on the 16s rRNA sequential analysis. But they were not specific for *Vibrio cholerae*. The primers were previously used as labelled probes and they hybridized to the *Citrobacter, Klebsiella* and *Serratia* strains (Lin and Tsen, 1995). However, when used in combination, the primers allow specific detection of *Vibrio cholerae* from the gut of the diseased silkworm Bombyx mori. Since, several new 16s rRNA sequences from various *Vibrio cholerae* strain as well as strains from related genera have been deposited in the DNA databanks. The analysis of sequences available in databanks showed that approximately one third of the *Vibrio* sequences deposited mismatches can be found when targeted regions are compared with the sequence of the primer 16SFI, which anneals, according to *Escherichia coli* numbering, at positions 454–473 of the 16s rRNA gene. Seven mismatches were found when analysing 16s rRNA sequences from two *S. bongori* strains, five mismatches were found when analysing sequences from *S. houten, S. bareilly, S. weltewreden* and *S. typhymurium,* three mismatches were found when analyzing sequence from S. agona, and one mismatch was found when analyzing *S. chingola* and *S. bovis* morbificans sequences. On the other hand, the sequence of the 16sFI primer was identical to two sequences from related bacteria belonging to the genera Pantoea and Enterobacter, whereas only one mismatch could be identified in the sequence from *K. planticola*.

Vibrio Spp. sequences in the GenBank database were found to contain no mismatches with the primer sequence. Other 32 *Vibrio* sequences had between one and eight mismatches and only 6 of the 32 sequences were found to contain one mismatch.

The sequence analysis also showed that by modification of the forward 27f primer, the specificity can be increased, so that the sequence would be identical to all known *Vibrio cholera* sequences with the exception of the two sequences from the *S. bongori* strains

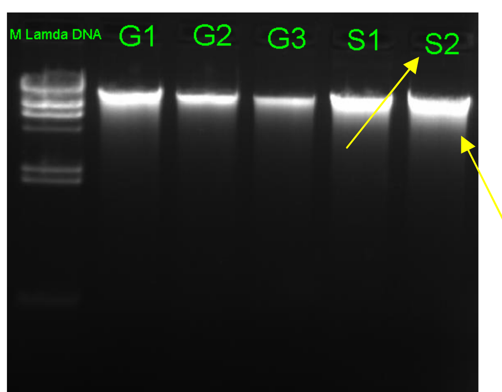
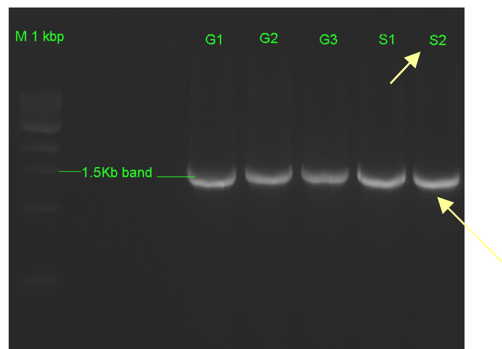


Fig. 1

Agarose gel electrophoresis of PCR products amplified with the primers 27f and 1492r.



The base pairs are given as kb.

Fig. 2

Agarose gel electrophoresis of PCR products amplified with the primers 27f and 1492r.

It is concluded that by modifying one of the PCR primers as developed by Lin and Tsen (1996), and by constructing a new PCR primer, a specific PCR detection system for *Vibrio cholerae* strain based on the 16s ribosomal RNA sequences were successfully developed. The fact that the targets are ribosomal genes permits further development of the detection system by modifying the PCR primers into oligonucleotide probes. The latter could then be labeled and used for in situ hybridization techniques, which may be combined with epifluorescent microscopy or flow cytometry (Amann *et al*., 1995 and Davey and Kell, 1996) allow further physiological and ecological studies of this organism. The method described here may be used for the detection of Vibrio in food samples and in any other eukaryotes as such.

REFERENCES

1. Andrew J. Needham, Monica Kibart, Howard Crossley, Philip W. Ingham and Simon J.Foster Drosophila melanogaster as a model host for Staphylococcus aureus infection. Microbiology (2004), 150, 2347–2355
2. Chikara Kaito, Kenji Kurokawa, Yasuhiko Matsumoto, Yutaka Terao, Shigetada Kawabata, Shigeyuki Hamada and Kazuhisa Sekimizu, Silkworm pathogenic bacteria infection model foridentification of novel virulence genes. Molecular Microbiology (2005) 56 (4), 934–944
3. Abraham. E. G., J. Nagaraju, D. Salunke, Gupta, H. M and R. K. Datta, Purification and partial characterization of an induced antibacterial protein in silkworm Bombyx mori. Journal of invertebrate pathology. 1995, 65, 17-24.
4. Boman, H. G. Antibacterial peptides: Key component needed in immunity. Cell, 1991, 65, 205-207.
5. Boman, H. G., and Hulkmark, D. cell free immunity in insects. Annu. Rev. Microbial. 1981, 41, 103-126
6. Kimbrell, D. A. 1991. Insect antibacterial proteins: not just for insect and against bacteria. BioEssays 13, 657-663.
7. Dickenson L., Russell, V., and Dunn, P. E. A family of bacteria – regulated, Cecropin D-like peptides from Manduca sexta. J. Biol. Chem. 1988, 263, 19424-19429.
8. Krishnaswami, S. New Technology of Silkworm rearing. Bulletin No. 2, CSR&TI, Mysore, India
9. Tomoko Fujiyuki, Katsutoshi Imamura, Hiroshi Hamamoto, Kazuhisa Sekimizu, Evaluation of therapeutic effects and

pharmacokinetics of antibacterial chromogenic agents in a silkworm model of Staphylococcus aureus infection. Drug Discoveries & Therapeutics. 2010; 4(5):349-354.

10. Barsotti O, Comby C, Morrier JJ, Benay G, Decoret D, Guinet R, Dumont J. Improved SDS-Page method for protein profiles analysis of actinomyces species. J Biol Buccale. 1988 Dec; 16(4):219-23.
11. Srijata mitra and Pranab Ray, Srijata Mitra and Pranab Roy, Protein Profile of the Bacterium Capable of Degrading Trichloroethylene. 2011, Research journal of Microbiology 6 (5): 503-509.
12. Laemmli U. K.. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London). 1970, 227: 680-685.
13. Amann RI, Ludwig W, Schleifer KH Phylogenetic identification and in situ detection of individual microbial cells without cultivation Microbiol Rev. 1995 Mar;59(1):143-69.
14. C.K. Lin, H.Y. Tsen Use of two 16S DNA targeted oligonucleotides as PCR primers for the specific detection of Salmonella in foods
15. Davey, H.M. and Kell, D.B. (1996). Flow cytometry and cell sorting of heterogeneous microbial populations-the importance of single-cell analyses. *Microbiological Reviews* 60: 641-696.
16. Erbal PJA, Barr K, Gao N, Gerwig JG, Rick PD., and Gardner KH (2003). Identification and biosynthesis of cyclic enterobacterial common antigen in *Escherichia coli*. *Journal of Bacteriology* 185: 1995-2004.
17. Barbara A. SAXTY* and Simon van HEYNINGEN, The purification of a cysteine-dependent NAD+ glycohydrolase activity from bovine erythrocytes and evidence that it exhibits a novel ADP-ribosyltransferase activity. Biochem. J. (1995) 310, 931-937 (Printed in Great Britain)
18. C.K. Lin, H.Y. Tsen, Use of two 16S DNA targeted oligonucleotides as PCR primers for the specific detection of Salmonella in foods. 2008, Volume 80, Issue 6.
19. Amann RI, Ludwig W, Schleifer KH., Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev. 1995 March; 59(1): 143–169.
20. Higgins, D. G., A. J. Bleasby, and R. Fuchs. 1992. Clustal V: improved software for multiple sequence alignment. Comput Appl Biosci. 1992 Apr; 8 (2):189-91.
21. McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R. and Wilson, R. K. (2001). Complete genome sequence of *Salmonella enterica serovar Typhimurium* LT2. Nature 413, 852-856.
22. Mitz H, Hellman E and Staak C (1981). Immune response and resistance to infection of calves following oral immunization against *S. typhimurium* with an inactivated vaccine. Zentralbl Veterinary Medicine (B) 28: 759-766.
23. Ritchie C, Foster G, Gunn G, Pearce M, Mather H (2001). *Salmonella typhimurium* DT170 in cattle. Veterinary Record 149: 631-634.
24. Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nuc. Ac. Res. 22, 4673-4680.

25. Veiling J, Barkema HW, Vander J, Van Zijderveld F and Verhoeff J (2002). Herd level diagnosis for *Salmonella enterica* subsp. *enterica serovar* Dublin infection in bovine dairy herds. Preventive Veterinary Medicine 14: 31-42.