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Identification and Phylogenetic Analyses of Two Isoforms of the Antibacterial Gene Diptericin from the Larval Tissue of *Musca domestica* (Diptera: Muscidae)

ORIGINAL INVESTIGATION

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ABSTRACT

Objective: Insect immune system has a potent arsenal of antimicrobial peptides (AMPs) that cooperate to clear microbial invasions. Here we aimed to explore the immune response of *Musca domestica* larvae when bacterially challenged and pick up induced antibacterial genes. These genes can be used in the production of novel antibiotics to compensate for the increasing demand of antibiotics in the era of resistant bacterial strains.

Materials and Methods: Hemolymph and whole body of third instar larvae were collected at 2-h intervals for 24 h postinfection. Integer and pure total RNA were transcribed into cDNA. Differential display technique was used to identify differentially expressed genes. Ten reproducible bacterial-induced bands were sequenced. Sequenced DNA fragments were deposited in GenBank under KM205630 and Hl205631 accession numbers.

Results: Sequence analyses indicated that two DNA fragments designated as *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ were identified as diptericin-related sequences, for which single open reading frame (orf) encoding 99 and 80 amino acids were detected, respectively. Signal peptide was predicted only for *Md*Dip$_{WB}$. Meanwhile, prosequence was predicted only for *MdDip*$_{HL}$. Calculated molecular masses of mature *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ were 8.8 and 6.97 Kilo Daltons (KDa), respectively. Propeptides of *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ were more stable than mature peptides. Comparing *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ nucleotide sequences, 26 substitutions and 4 deletions were observed in *Md*Dip$_{WB}$. Despite the 90% identity between *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ nucleotide sequences, no significant similarity was observed between their deduced amino acids. Nucleotide and deduced amino acids of *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ created significant similarity with other diptericins isolated from M. domestica. On comparing amino acid sequences of our putative polypeptides to their corresponding sequences, overexpression of many specific amino acid residues was observed.

Conclusion: Our findings suggested that *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ are two isoforms of the same gene.

Keywords: AMPs, diptericin, *musca domestica*, diptera, immune response

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INTRODUCTION

Despite their lack of adaptive immunity, insects protect themselves via a powerful innate immune system. Induction of the innate immune system of insects resulted in a wide range of responses (cellular and humoral) corresponding to the inducer. Humoral responses contain melanization and synthesis of AMPs. Insect immune responses are based on recognition of the pathogen as nonself and induction of suitable genes and biochemical pathways that result in the production of a potent arsenal of low molecular weight AMPs (1, 2). These AMPs are produced by fat body and certain blood cells and released in hemolymph (3, 4). AMPs were classified into three broad types: (i) linear peptides forming α-helices and deprived of cysteine residues, *e.g.*, cecropins; (ii) cyclic peptides containing cysteine residues, *e.g.*, defensins and attacins; and (iii) peptides with an overrepresentation in proline and/or glycine residues, *e.g.*, lebocins and moricins (5). AMPs are positively charged small amphipathic molecules (possessing both hydrophobic and hydrophilic regions). Physicochemically, they are strong cationic [isoelectric point (PI) 8.9-10.7] and heat-stable (100 °C, 15 min) molecules with no drug fastness and no effect on eukaryotic cell (6, 7). Identification and isolation of these AMPs and determination of their primary structures or DNA sequences are of vital importance, both to the study of non-specific immune response mechanism of insect against pathogen invasion and the application of these substances in the biopharmaceutical industry that will ultimately benefit mankind (8-11).

The house fly *Musca domestica* is a cosmopolitan medical insect considered to have a highly effective immune defense mechanism as it is rarely infected even when reared in large-scale, high-density conditions (12-18). To date, hundreds of AMPs have been described in insects. However, there are few reports on the isolation, purification, and molecular identification of AMPs from the house fly larvae, including lysozyme, attacin, cecropin, diptericin, and defensin.

Therefore, the main objectives of the present study are to investigate immune responses of the house fly larvae when bacterially challenged at different time intervals and pick up the induced genes. Herein we report the isolation, se-

quence characterization, and phylogenetic analysis of two isoforms of the antibacterial gene diptericin from two larval tissues of *M. domestica*. This study is the first step toward the discovery of a new antibiotic, in response to the growing trend of bacterial resistance.

MATERIALS and METHODS

Insects and bacterial strains

A laboratory colony of the house fly *M. domestica* used for our experiments was originally obtained from the Research Institute of Medical Entomology, Dokki, Giza, Egypt, and maintained in the insectary of the Department of Zoology, Faculty of Science, Menoufia University (27±2 °C and 70±5% Relative humidity (RH) and 14/10 light/dark photoperiod cycle), according to Hashem and Youssef (19).

One gram-positive *Streptococcus sanguinis* and one gram-negative *Proteus vulgaris* were obtained from the Unit for Genetic Engineering and Agricultural Biotechnology, Faculty of Agriculture, Ain Shams University, and used for insect immunization. Bacteria were grown in a peptone medium (1%), supplemented with 1% meat extract and 0.5% NaCl, at 37 °C in a rotary shaker.

Bacterial challenge, hemolymph, and larvae collection

Bacterial challenge was performed by injecting 300-500 newly molted third instar larvae with 2 µL of approximately 1×10^6 (cells/mL) log phase bacteria dissolved in membrane-filtered saline using a sterile thin-needled microsyringe. Bacterial strains were used for immunization separately and in combinations. Hemolymph and third instar larvae were collected at 2-h intervals for 24 h postinfection and stored at −80 °C for a week. Hemolymph was collected in aliquotes (100 µL each) by cutting off the anterior tip of the larvae with sterile fine scissors. Hemolymph was collected in an ice-cold eppendorf containing few crystals of phenylthiourea to prevent melanization. Larvae were collected intact and stored as previously mentioned. The same procedures were applied to the control group, with the difference that it was injected with saline without bacteria. All necessary permits for this study were obtained from the local ethics committee of Cairo University. This study did not involve endangered or protected species. The informed consent rules are not applicable for this study.

DD-PCR using primers corresponding to well-known defense genes

Total RNA of hemolymph and larvae was extracted using RNeasy kit according to the manufacturer's instructions (Qiagen, Germany). Residual genomic DNA was removed from RNA using RNase-free DNase (Ambion, Germany). RNA was dissolved in DEPC-treated water, quantified using a BioPhotometer 6131 (Eppendorf, Germany), and analyzed on 1.2% denatured agarose gel to ensure its integrity. The 260/280 and 260/230 ratios were examined for protein and solvent contamination.

A total of 100 ng of DNA-free total RNA was converted into cDNA using a mix of random and oligodT_{20} primers according to the ABgene protocol (ABgene, Germany). The first cDNA strand was synthesized in a thermal cycler (Eppendorf, Mastercycler 384, Germany) programmed at 42 °C for 1 h and 72 °C for 10 min and a soak at 4 °C. The cDNA was aliquoted and stored at −80 °C until processed (within a week). A total reaction volume of 25 µL [containing 2.5 µL PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1 U *Taq* DNA polymerase (AmpliTaq, Perkin-Elmer, USA), 2.5 µL of 10 pmol primer (Table 1), and 2.5 µL of each cDNA] was cycled in a DNA thermal cycler (Eppendorf, Mastercycler 384, Germany). The amplification program was one cycle at 94 °C for 5 min (hot start), followed by 40 cycles at 94 °C for 1 min, 36 °C for 1 min, and 72 °C for 1 min. The reaction was then incubated at 72 °C for 10 min for final extension. PCR product was visualized on 2% agarose gel and photographed using gel documentation system. For DNA contamination assessment, a no-reverse transcription control reaction was performed.

Table 1. List of primer names and their nucleotide sequences used in the study

No	Name	Sequence
1	OP-A07	5' GAA AGG GGT G 3'
2	OP-A12	5' GTG ATC GCA G 3'
3	OP-A18	5' AGG TGA CCG T 3'
4	OP-AX06	5' AGG CAT CGT G 3'
5	OP-C01	5' TTC GAG CCA G 3'
6	OP-C04	5' CCG CAT CTA C 3'
7	OP-C19	5' GAC GGA TCA G 3'
8	OP-E19	5' ACG GCG TAT G 3'
9	OP-M17	5' GTT GGT GGC T 3'
10	OP-Q18	5' GGG AGC GAG T 3'
11	OP-P10	5' GAG AGC CAA C 3'

Ten reproducible bacterial-induced bands were eluted, cloned in *PCR-TOPO* vector (Invitrogen, USA), and sequenced using M_{13} universal primer. Sequencing was performed using T^7 Sequencing™ kit (Pharmacia, Biotech, USA) and model 310 automated sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide and deduced amino acid sequences were analyzed using EditSeq-DNAstar Inc., Expert Sequence Analysis software, Windows 32 Edit Seq 4.00 (1989-1999), and ExPasy database (http://expasy.org/tools/dna.html). Blast search for alignment of the obtained sequence with the published ones was performed using the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition to the above mentioned analyses, ExPasy Proteomics Server (http://expasy.org/tools) was used to calculate the physicochemical parameters of the translated peptide (ProtParam tool). Furthermore, primary and secondary structural analyses, posttranslational modifications, and topology predictions were investigated using SignalP, NetCGlyc, NetOGlyc, NetGlycate, YinOYang, NetPhos, NetPhosK, Sulfinator, ProP, NetNES, TatP, and TMHMM tools. Phylogenetic analyses of the nucleotide sequence and its deduced amino acids were performed using the Phylogeny.fr web service, One Click mode. Poorly aligned positions and divergent sequences were manually eliminated. Multiple alignments of published diptericins and diptericin-related nucleotide sequences were performed before phylogenetic analyses to manually estimate sequence lengths. A 100% homology in the sequences of the same species with different accession numbers were represented by only one sequence. The cloned DNA fragment was deposited in GenBank under the KM205630 and Hl205631 accession numbers.

RESULTS

Differential display

As the identification of induced antibacterial genes was the main objective of this study, differential display technique was used to characterize the genetic variation (at RNA level) between bacterially challenged and control *M. domestica* third instar larvae.

Whole body and hemolymph samples were differentially displayed at 2-h intervals for 24 h postinfection with *S. sanguinis*, *P. vulgaris*, and a combination of both strains. It was observed that the challenged insects died after 24 h postinfection. Figures 1 and 2 show the results of differentially displayed cDNAs of the control and bacterially challenged insects using 11 decameric arbitrary primers. The total number of bands (transcripts) resolved in 2% agarose gel for both control and bacterially challenged insects was 85 bands with molecular size >1400 to ~180 bp. Sixty-two polymorphic bands were differentially displayed with the used primers. The reproducible bands indicated by arrows in Figures 1 and 2 were eluted, cloned, and sequenced using M_{13} universal primer. Two DNA fragments designated as *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ were identified as diptericin-related sequences.

Nucleotide sequence and sequence analyses

Nucleotide sequences of *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ and their deduced amino acid sequences are shown in Figures 3 and 4. A single *orf* that could encode a polypeptide of 99 and 80 amino acids was detected for *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$, respectively. One stop codon was found at the 3' end of both sequences. The flanking region of the initiation codon ATG was AAAATGCAA for *Md*Dip$_{WB}$ and CCGATGATA for *Md*Dip$_{HL}$. The lengths of 3' untranslated regions were 74 and 62 bp before the poly(A) track for *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$, respectively (Figure 3, 4). One polyadenylation sequence AATAAA was located 40 bp downstream from the stop codon of *Md*Dip$_{WB}$ (Figure 3). Meanwhile, two putative polyadenylation sequences AATAAA were located 3 and 32 bp downstream from the stop codon of *Md*Dip$_{HL}$ (Figure 4). Signal peptide sequence was predicted for *Md*Dip$_{WB}$ but not for *Md*Dip$_{HL}$. Meanwhile, prosequence was predicted for *Md*Dip$_{HL}$ and not for *Md*Dip$_{WB}$. The deduced *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ polypeptides contained 13 and 13 basic, 9 and 5 acidic, 34 and 39 hydrophobic, and 33 and 34 polar amino acids, respectively. The calculated molecular masses of the full-length diptericins were 10.9 and 8.8 KDa for *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$, respectively. Meanwhile, the calculated molecular masses of the mature diptericins of *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ were 8.8 and 6.97 KDa, respectively. The calculated PIs of the full-length *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ were 7.79 and 9.77, respectively. The calculated PIs of the mature peptides were 8.2 and 5.9 for *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$, respectively. The net charges of the full-length and mature peptides of *Md*Dip$_{WB}$ at pH 7.0 were 1.2 and 1.3, respectively. Meanwhile, the net charges of the full-length and mature peptides of *Md*Dip$_{HL}$ at pH 7.0 were 3.4 and 0, respectively. The propeptides of *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ were more stable (instability index (II): 30.38 and 29.16) than their mature peptides (II: 38.03 and 26.98). The ratios of hydrophilic residues were 22% and 28% for the propeptide and mature peptide of *Md*Dip$_{WB}$. These ratios were 22% and 15% for the propeptide and mature peptide of *Md*Dip$_{HL}$. On the other hand, the ratios of hydrophobic residues were 37% and 51% for the propeptides of *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$, respectively. These ratios were 29% and 58% for the mature peptides of *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$, respectively.

On comparing *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ nucleotide sequences to each other, 26 substitutions (2 CT, 2 GA, 3 TA, 3 TC, 4 AC, 2 AG, 3 GT, CA, 2 AT, 2 TG, GC, TC) and 4 deletions (ATGA) were observed in *Md*Dip$_{WB}$ (Figure 5). A segment of 57 nucleotides (signal peptide) was observed in *Md*Dip$_{WB}$. Despite the 90% identity between *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ nucleotide sequences, no significant similarity was observed between them on the basis of amino acid sequences (Figure 6).

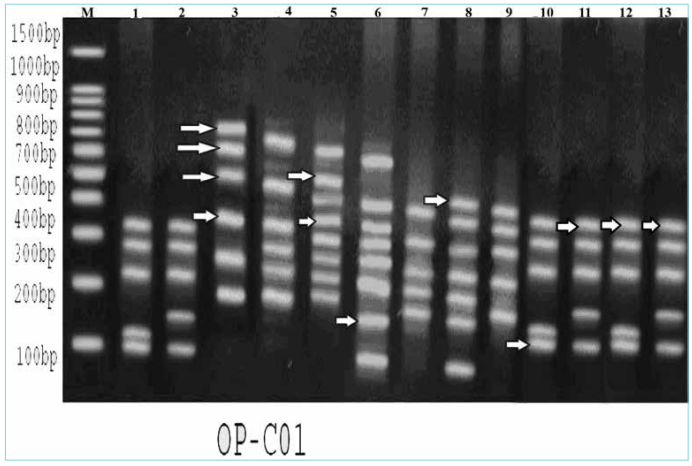

Figure 1. Representative 2% agarose gels of DD-PCR patterns generated from control and bacterially challenged whole body samples using 11 primers. Lane M: DNA marker 100 bp; Ladder, Lane1: control; and lanes 2-13: treated larvae at 2-h intervals for 24 h postinfection. Arrows refer to differentially displayed sequenced bands.

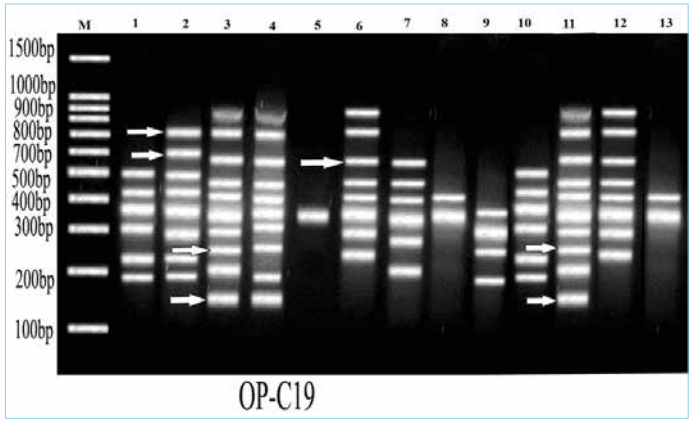

Figure 2. Representative 2% agarose gels of DD-PCR patterns generated from control and bacterially challenged hemolymph samples. Lane M: DNA ladder 100 bp; lane1: control; and lanes 2-13: treated larvae at 2-h intervals for 24 h postinfection. Arrows refer to differentially displayed sequenced bands.

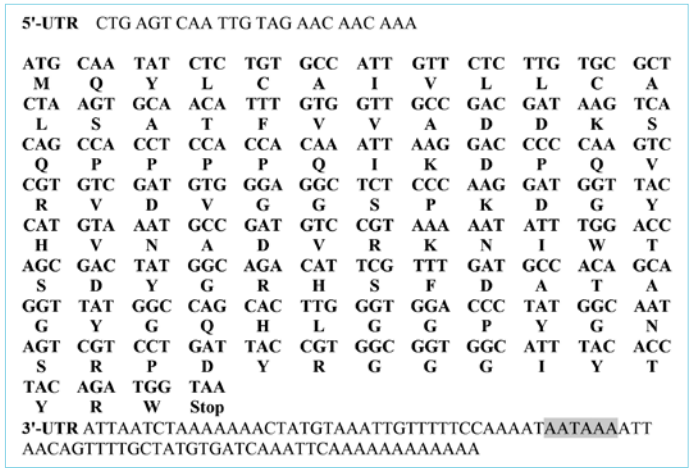

Figure 3. Nucleotide and corresponding deduced amino acid sequence of *Musca domestica* whole body diptericin gene (*Md*Dip$_{WB}$)

On comparing the present diptericin nucleotide sequences *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ with other diptericins isolated from *M. domestica* (Acc# FJ794602, FJ795370, and FJ748596), only 29 different nucleotides were observed throughout the five sequences, regardless of the first 61 nucleotides which were deleted from the *Md*Dip$_{HL}$ sequence (Figure 7).

In addition, the nucleotide and deduced amino acid sequences of *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ were blasted to all diptericin-related sequences in GenBank database. A blast search of putative *Md*Dip$_{WB}$ peptide created significant alignment with 26 insect-published peptide sequences (25 diptericins and 1 attacin). The *Md*Dip$_{WB}$ putative peptide exhibited 97% and 94% identity with *M. domestica* diptericins (Acc# ACO35257 and ACN93798, respectively), 72% with *Glossina morsitans* diptericin (Acc# AAL34111), and 59% identity with *Stomoxys calcitrans* diptericin (Acc# AAY98016). The percentage identity of *Md*Dip$_{WB}$ putative peptide ranged from 97% to 34% for diptericin (Acc# ACO35257 and BAM63553) and 29% for attacin (Acc# ABS18285). Meanwhile, the *Md*Dip$_{WB}$ nucleotide sequence created significant identity with 20 insect-related diptericins.

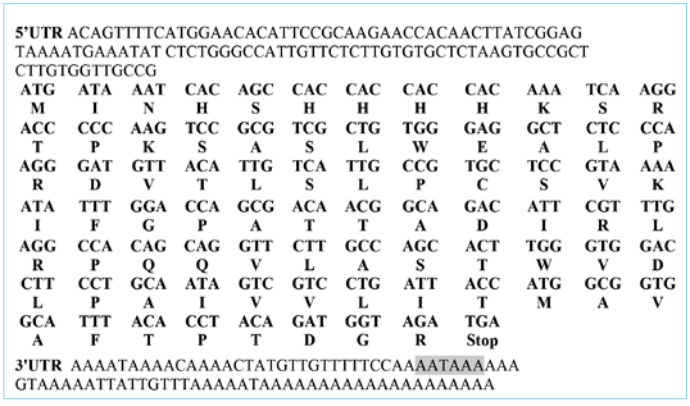

Figure 4. Nucleotide and corresponding deduced amino acid sequence of *Musca domestica* hemolymph diptericin gene (*Md*Dip$_{HL}$)

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MdDipWB      ATGCAATATCTCTGTGCCATTGTTCTCTTGTGCGCTCTAAGTGCAACATTTGTGGTTGCC 60
MdDipHL      ------------------------------------------------------------

MdDipWB       GACGATAAGTCACAGCCACCTCCACCACAAATTAAGGACCCCCAAGTCCGTGTCGATGTG 120
hemolymph    -ATGATAAATCACAGCCACCACCACCACAAATCAAGGACCCCCAAGTCCGCGTCGCTGTG 59
              * ***** ********** ********** ***************** **** ****

MdDipWB       GGAGGCTCTCCCAAGGATGGTTACCATGTAAATGCCGATGTCCGTAAAAATATTTGGACC 180
hemolymph    GGAGGCTCTCCCAAGGGATGTTACATTGTCATTGCCGTGCTCCGTAAAAATATTTGGACC 119
             ****************   *****  *** * *****   ********************

MdDipWB       AGCGACTATGGCAGACATTCGTTTGATGCCACAGCAGGTTATGGCCAGCACTTGGGTGGA 240
hemolymph    AGCGACAACGGCAGACATTCGTTTGAGGCCACAGCAGGTTCTTGCCAGCACTTGGGTGGA 179
             ****** * ***************** ************ * *****************

MdDipWB       CCCTATGGCAATAGTCGTCCTGATTACCGTGGCGGTGGCATTTACACCTACAGATGGTAA 300
hemolymph    CCTTCCTGCAATAGTCGTCCTGATTACCATGGCGGTGGCATTTACACCTACAGATGGTAG 239
             ** *   ********************** ******************************

MdDipWB         ----
hemolymph      ATGA 243
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Figure 5. Comparison of *MdDip$_{WB}$* and *MdDip$_{HL}$* nucleotide sequence from *Musca domestica*

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MdDipWB       MQYLCAIVLLCALSATFVVADDKSQPPPPQIKDPQVRVDVGGSPKDGYHVNADVRKNIWT 60
hemolymph     -MINHSHHHHKSRTPK-SASLWEALPRDVTLSLP-CSVKIFG-PATTADIRLRPQQ-VLA 55
                  :     : :..  .:  :: *    :. *   *.: * *    .:.   :: : :

MdDipWB       SDYGRHSFDATAGYGQHLGGPYGNSRPDYRGGGIYTYRW 99
hemolymph     STW--VDLPAIVVL-ITMAVAFT---P--TDGR------ 80
             *: .:*.  :.:  * .*
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Figure 6. Comparison of *MdDip$_{WB}$* and *MdDip$_{HL}$* nucleotide sequence from *Musca domestica*

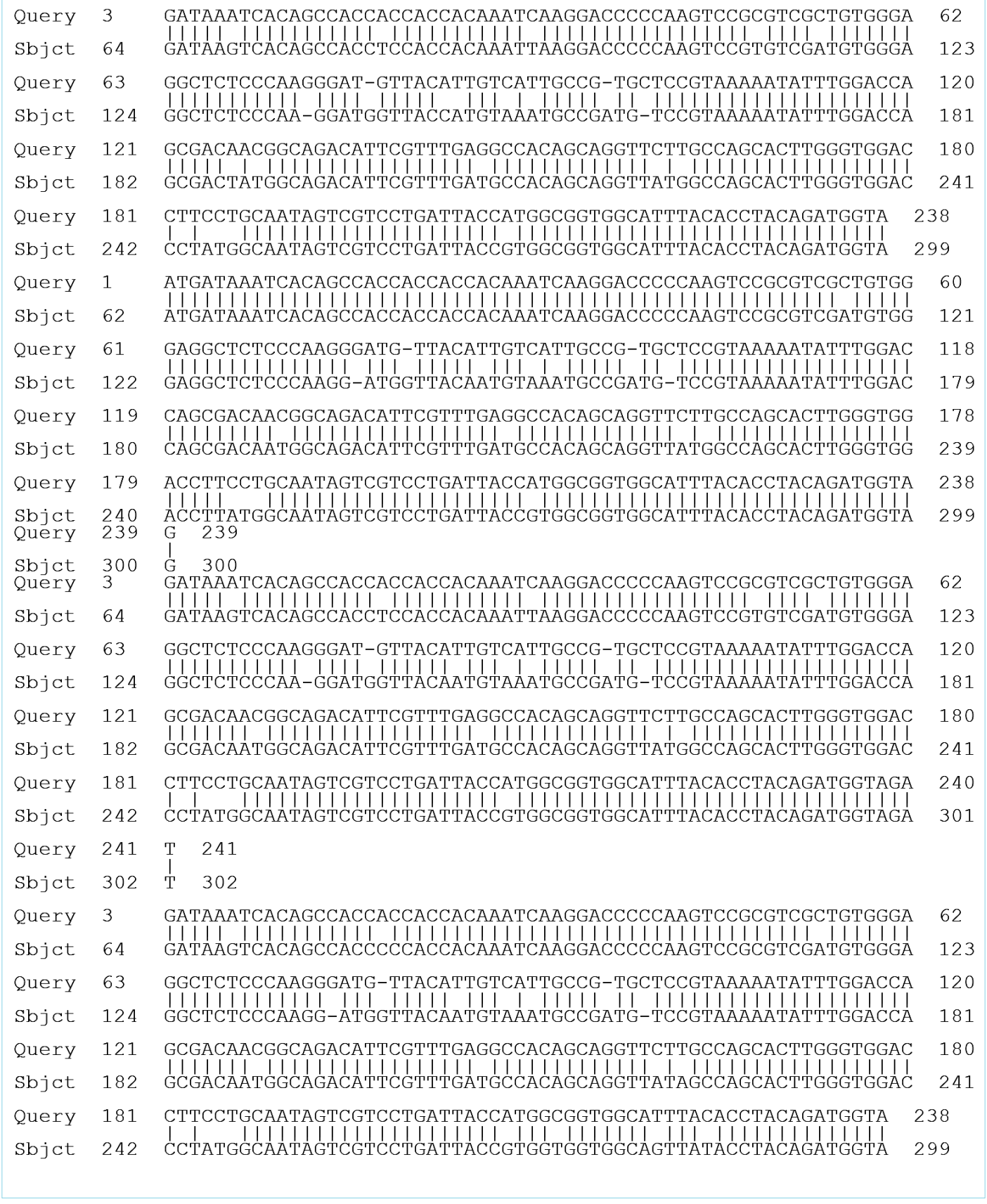

Figure 7. Comparison of $MdDip_{WB}$ and $MdDip_{HL}$ nucleotide sequence with other diptericins isolated from *Musca domestica*

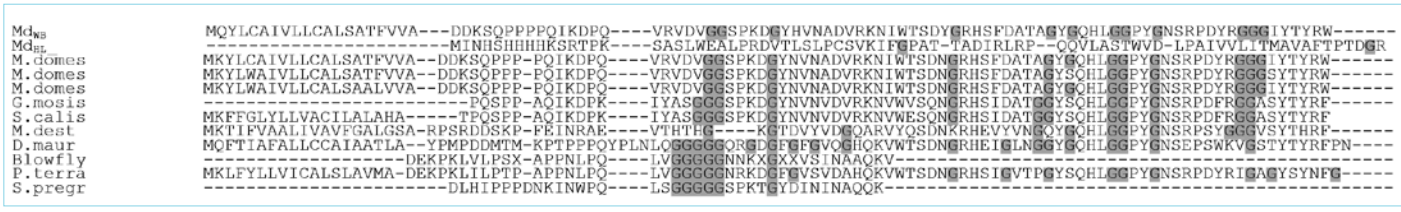

Figure 8. Alignment of *MdDip*$_{WB}$ and *MdDip*$_{HL}$ deduced amino acid sequence with other diptericins

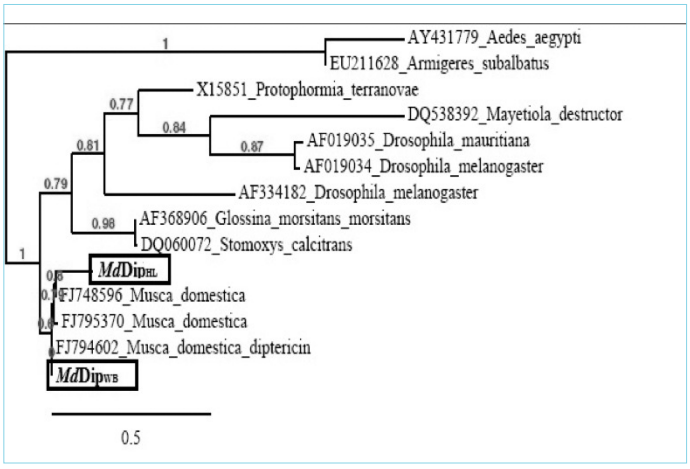

Figure 9. Phylogenetic analysis of *MdDip*$_{WB}$ and *MdDip*$_{HL}$ nucleotide sequences compared to sequences registered in NCBI

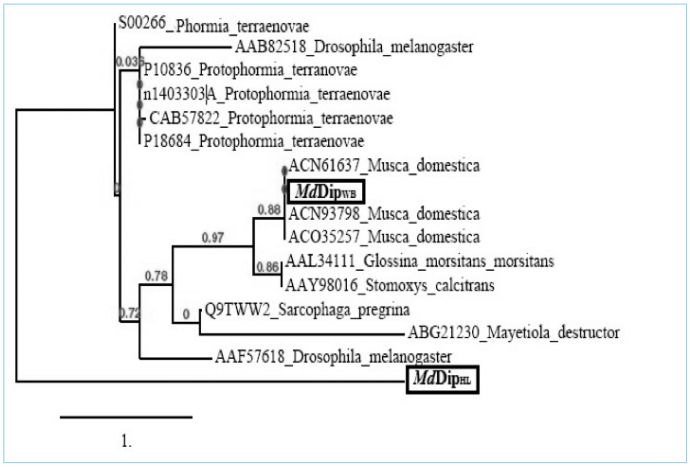

Figure 10. Phylogenetic analysis of *MdDip*$_{WB}$ and *MdDip*$_{HL}$ deduced amino acid sequences compared to sequences registered in NCBI

On comparing amino acid sequence of our putative polypeptides *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ to their corresponding sequences of *M. domestica*, *G. morsitans*, *S. calcitrans*, *Mayetiola destructor*, *D. mauritiana*, blow fly, *Protophormia terraenovae*, and *Sarcophaga peregrina* (Acc# ACO35257, ACN61637, ACN93789, AAL34111, AAY98016, ABG21230, AAB82532, S00266, P18684, and Q9TWW2, respectively), 8-19 overexpressed glycine residues were observed throughout the compared sequences, excluding *Md*Dip$_{HL}$ which exhibited overexpression of other residues (Fig. 8). In addition to glycine residues, *Md*Dip$_{WB}$ overexpressed Asp (9), Tyr (8), Pro (8), and Ala (7) residues. *Md*Dip$_{HL}$ showed overexpression of Thr (8), Ala (8), Leu (8), Pro (7), Ser (7) and Val (7), but not of Gly (2). These are comparable to other AMPs which exhibited overexpression of specific amino acid residue.

Primary, secondary structural analyses, posttranslational modifications, and topology predictions revealed that there was a signal peptide cleavage site between positions 20 and 21 for *Md*Dip$_{WB}$ and a propeptide cleavage site between positions 15 and 16 for *Md*Dip$_{HL}$. One potential glycated lysine was predicted at position 57 for *Md*Dip$_{WB}$ and at position 10 for *Md*Dip$_{HL}$. Five leucine-rich nuclear export signals (NES) were predicted at positions 9, 10, 11, 12, and 13 for *Md*Dip$_{WB}$, whereas only one leucine-rich NES was predicted at position 31 for *Md*Dip$_{HL}$. No O-glycosylation site was predicted for *Md*Dip$_{WB}$, whereas four O-glycosylation sites were predicted at positions 28, 42, 75, and 77 for *Md*Dip$_{HL}$. Seven phosphorylation sites (Ser: 3 at positions 43, 67, and 85; Thr: 1 at position 96, Tyr: 3 at positions 48, 63, and 89) and 6 (4 S, 1 y, and 1 T) kinase-specific phosphorylation sites (highest score: 0.70 PKC at position 97) were predicted for *Md*Dip$_{WB}$, whereas 5 phosphorylation sites (Ser: 3 at positions 16, 18 and 30 and Thr: 2 at positions 13 and 43) and 12 (7 S and 5 T) kinase-specific phosphorylation sites (highest score: 0.82 PKC at position 35) were predicted for *Md*Dip$_{HL}$. One transmembrane helix (21 aa. length: 53-74) and three beta-turns (positions: 28, 42, and 75-77) were predicted for *Md*Dip$_{HL}$.

Phylogenetic analyses of the *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ sequences

Phylogenetic analyses were performed on the *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ nucleotide sequences and their deduced polypeptides and the results of these analyses are shown in Figure 9 and 10. In the case of nucleotide sequence, a phylogenetic tree was generated from 14 diptericin-related sequences (8 dipteran species) by neighbor-joining distance analysis with maximum sequence difference 1.0 (Figure 9). The topology shows two distinct lineages including two diptericins from family: Culicidae (lineage I) and 12 diptericins from families Calliphoridae, Cecidomyiidae, Drosophilidae, Glossinidae, and Muscidae (lineage II). The maximum nucleotide sequence divergence was exhibited in the second lineage (5 phylogenetic groups). Meanwhile, the diptericin sequences appear in the other lineage as one phylogenetic group. *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ were clustered with the other three *M. domestica* diptericins (Acc# FJ748596, FJ795370, and FJ794602) in a monophyletic sister clade (Figure 9). Meanwhile, the other muscid sequence (*Stomoxys*) was grouped with *Glossina* sequence in a separate sister clade (Figure 9). In the case of *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ deduced amino acid sequences, a phylogenetic tree was generated from sequence data of 16 published sequences (8 dipteran species) by neighbor-joining distance analysis with maximum sequence difference 0.97 (Figure 10). The topology shows two distinct lineages including 15 diptericins from the families Calliphoridae, Cecidomyiidae, Drosophilidae, Glossinidae, Sarcophagidae, and Muscidae (lineage I) and *Md*Dip$_{HL}$ (lineage II). The maximum amino acid sequence divergence was exhibited in the first lineage (7 phylogenetic groups). Meanwhile, our hemolymph diptericin sequence (*Md*Dip$_{HL}$) appeared in the other lineage as one phylogenetic group. *Md*Dip$_{WB}$

was clustered with the other three *M. domestica* diptericins (Acc# ACO35257, ACN61637, ACN93789) in a monophyletic sister clade (Figure 10). Meanwhile, the other muscid sequence (*Stomoxys*) was grouped with *Glossina* sequence in a separate sister clade (Figure 10). Generally, clustering diptericins from different dipteran families in monophyletic sister clade is a very strong clue that insect diptericins may share a common ancestor (Figure 10).

DISCUSSION

The main objective of the current work is to isolate and characterize antibacterial genes from the house fly *M. domestica* after bacterial challenge. To accomplish this objective, third instar larvae were injected with gram-positive bacteria (*S. sanguinis*), gram-negative bacteria (*P. vulgaris*) and combination of the two types (mix). The aim of such injection was to trigger the immune system of the insect which possesses a range of defense mechanisms to effectively combat bacterial invasion.

DD-PCR technique is considered a powerful genetic screening tool for complicated dynamic tissue processes, particularly when multiple, limited-sized samples are involved, because it allows for simultaneous amplification of multiple arbitrary transcripts (20). This technique was developed as a tool to detect and compare altered gene expression in eukaryotic cells (21), screen mRNAs, and characterize differentially expressed mRNAs (22-25).

In the present study, the mRNA display pattern of normal unchallenged larvae was compared with that of bacterially challenged larvae of the house fly *M. domestica*. To produce a differential display, reverse transcription PCR amplifications were performed. DD-PCR study revealed that several common bands were observed in both control and challenged samples (housekeeping genes). Very few bands were recorded in control insects and disappeared in challenged ones (genes were turned off). On the other hand, many bands were induced as a result of bacterial challenge at different time intervals postinfection.

Many studies have described the enhancement of the insect immune system and induction of AMPs due to stress and/or bacterial challenge (26-33). Elution and sequencing of the induced bands were performed and the generated sequences were blasted to defensin, diptericin, and attacin sequences.

The humoral immune system mainly relies on antibacterial polypeptides such as the diptericin and attacin-like proteins. Diptericin genes were isolated from the order Diptera. The length of diptericin varies from 433 bp in *M. domestica* (Acc# FJ748596) and 466 bp in *D. melanogaster* (Acc# M55432) to 435 bp in *P. terranovae* (Acc# X15851). The *orf* of *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ (300 and 243 bp, respectively) was comparable in size to that of other *Musca* diptericin genes (297 and 300 bp for Acc# FJ748596 and FJ794602, respectively). Reconstruction of the phylogenetic trees of the *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ nucleotide sequences and their deduced polypeptides resulted in two different topologies. In spite of constructing two different topologies, both trees clustered the *Md*Dip$_{WB}$ and *Md*Dip$_{HL}$ sequences with that of *M. domestica* to indicate that they descend from a common ancestor. The grouping of *M. domestica* in one sister clade indicated that they may be homologous or share some similarity. In addition, the diptericin-like sequences were diverged in many sister clades as nucleotides but they were clustered in a monophyletic group as amino acids due to the difference in codon usage in the different insect species.

Diptericins, found in several insects, contain one P- and one G-domain (34). All members of this family are active against a limited number of gram-negative bacteria. Drosocin and pyrrhocoricin share a great deal of sequence homology with the N-terminal 21-residue domain of yet another insect antibacterial peptide diptericin, isolated from *P. terranovae* (35, 36). In contrast to the medium-sized drosocin and pyrrhocoricin, diptericin consists of 82 amino acid residues with C-terminal sequence similarity to the glycine-rich proteins, namely attacins (37). The high glycine content and presence of the pentaglycine segment led to the assignment of diptericins to the attacin rather than the apidaecin peptide family. A diptericin analog has been isolated from *S. peregrina* (38), and a third diptericin sequence has been deduced from the cDNA of *D. melanogaster* (34). The *Phormia* diptericin carries two carbohydrate side-chains, one in the proline-rich domain attached to the same threonine that is glycosylated in drosocin and pyrrhocoricin and another in the glycine-rich domain. Although a number of close homolog *Phormia* diptericins can be isolated with different carbohydrate lengths, at least one monosaccharide is attached to all of these molecules (36, 39). Treatment of a diptericin variant containing two disaccharides with O-glycosidase resulted in the loss of antibacterial activity (36). This finding seems surprising as the otherwise similar *Sarcophaga* diptericin is potent, yet lacks any carbohydrate side-chains (38). All these uncertainties about diptericin called for a detailed structural activity study, especially as diptericin, unlike drosocin and pyrrhocoricin, is active on both solid-phase and liquid antimicrobial assays.

In conclusion, defense peptides and proteins constitute key factors in insect humoral immune response against invading microorganisms. It is generally assumed that each insect species possesses its own set of AMPs synthesized in response to nonself recognition. In this study, we characterized two diptericin isoforms, which appeared in larval whole body and hemolymph after bacterial challenge. They comprise a part of the defense peptide repertoire of *M. domestica*.

Such antibacterial genes had bactericidal activity when tested *in vitro* against standard microorganisms. However, pharmacological standardization and clinical evaluation of their effects are essential before using as a preventive and curative measure to common diseases related to the tested bacterial species. The isolated polypeptide fractions are further subjected to amino acid characterization and NMR spectrum and to estimate their concentration in the hemolymph. In spite of all the positive facts associated with AMPs, there have been a few problems. First, there are fewer data available on the unknown *in vitro/in vivo* toxicities of these peptides. Second, the stability of the synthesized compound formulations *in vivo* has not been studied in detail. Last, the cost of the production of these peptides on a large scale has been a major obstacle for quite some time. Hence, further studies should focus on identifying more such novel peptides, redesigning the existing peptides to get rid of their toxicity, and developing novel recombinant protocols to obtain greater yield of peptides at a lower cost.

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