

THE ANTIBACTERIAL EFFECT OF DRAGONFLY LARVAE *DIPLOCODES TRIVIALIS* (RAMBUR) HAEMOLYMPH ON THE FOOD BORNE PATHOGENS IN RAW MEAT

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Abstract - In our study, the hemolymph of Dragonfly Larvae *Diplocodes trivialis* (Rambur) which is considered as an alternative resource for antibiotics was tested against three bacterial strains, and it was found that three out of seven strains were sensitive to hemolymph. The sensitive bacterial strains were *Sallmonella typhimurium*, *Escherichia coli*, *Campylobacter jejuni*. Our results showed that dragon fly larvae hemolymph had antibacterial effects. This is the first study about antibacterial effects of *Diplocodedes trivialis* (Rambur) hemolymph.

Key Words: (Hemolymph, *Diplocodes trivialis*, Antibacterial Agents, Immunity, Bactreial strains and Media, Plate growth-inhibition assay)

1. INTRODUCTION

In this context, there is an urgent need for a new generation of antibiotics to complement the panel of drugs that are available to the clinicians and to provide new tools for multi-therapy treatment. One of the major areas of interest for the discovery and study of new antibiotics was the investigation of antimicrobial peptides derived from insect immune defense reactions. Defense peptides are key factors in innate immunity against bacteria and fungi in vertebrates as well as invertebrates. Particularly, in insects which lack an adaptive immune system, antimicrobial peptides play a crucial role in fighting against invading pathogens. In insects, systemic infection induces the synthesis of combinations of antibacterial peptides that are secreted from the immune organs, mainly the fat body, an analogue of the mammalian liver, into the haemolymph, where they reach high concentrations. The antibiotic activities have been either determined in vitro by using peptides directly purified from flies or produced in heterologous systems, or deduced by comparison with homologous peptides isolated in other insect species: Drosomycin and Metchnikowin show antifungal activity (Fehlbaum *et al.*, 1994; Levashina *et al.*, 1995); Cecropins have both antibacterial and antifungal activities (Ekengren and Hultmark, 1999); Drosocin and Defensin are predominantly active against Gram-negative and -positive bacteria, respectively (Dimarcq *et al.*, 1994; Charlet, *et al.*, 1999); and Attacins and Dipterocins are similar to peptides from other insects that show antibacterial activity (Asling, *et al.*, 1995). Insects are known to have both cellular and humoral immune systems which together form a potent defence against invading bacteria (Kimbrell, 1991). While humoral responses mainly involve the production of a variety of antibacterial proteins that are induced or increased in many insects. These antibacterial proteins can be categorized by homology as belonging to one of the several families, namely, the cecropins (Kylsten *et al.*, 1990; Samakovils *et al.*, 1990; 1991), attacins (Casteels *et al.*, 1990; Wicker *et al.*, 1990; Sun *et al.*, 1991) and defensins (Lambert *et al.*, 1989; Dimarcq *et al.*, 1990). Studies on these inducible antibacterial proteins in invertebrates seem to be important from the view point of comparative immunology. Due to this reason haemolymph of dragonfly larvae was selected as test tissue to study the antibacterial activity.

2. MATERIALS AND METHODS

The final instar larvae of dragonfly *Diplocodes trivialis* were used as the test animal and the raw meat samples were used as test material for microbial analysis.

2.1 Collection and maintenance of experimental larvae

The dragonfly larvae were collected manually from their natural breeding places, acclimatized and maintained at the laboratory conditions by following the method outlined by Varadaraj (1978). The larval instars were reared individually in separate plastic containers. As cannibalism is prevalent among the larvae, they were placed individually in plastic containers of 5 litre capacity (Fischer, 1961; Corbet, 1962; Prichard, 1964). Three quarters full of dechlorinated tap water and an inch square of cheese cloth and toothpicks were offered as foothold to the larvae. All the containers were examined regularly to observe whether moulting has taken place. The dragonfly larvae were fed *ad libitum* with mosquito larvae (Plate. I).



Fig -1: Maintenance of experimental larvae

2.2 Identification of larval instars

Various larval instars were identified by the morphometry of body length and head width by using the method of Dunkle (1985). The body length of the larvae was measured using a metric ruler from the proximal edge of the tip of the head to the dorsal tip of the Epiproct. The head width was measured across the widest part of the eyes using ocular micrometer fitted to a dissection microscope. The final instar larva with body length of 20 mm and head width of 8mm (Plate.II) were selected for the present investigation.



2.3. Meat sample preparation

Twenty-five grams of collected meat samples were weighed and transferred to sterile flasks containing 10 ml of phosphate buffer saline (PBS). Samples were homogenized using a meat grinder under aseptic conditions and was inoculated in different selective media such as Nutrient agar (MSA), (EMB) *E.coli*, MacConkey, Cetramide agar, SS agar, Karmail agar and Chromogenic media for *E.coli*, *S.typhimurium*, *Campylobacter* and *Enterococcus* respectively.

2.4. Antibiotics used for the assay

Cotrimoxazole, Centriaxone, Nitrofurantoin, Ciprofloxain, Chloramphenicol, Ampicillin, Tetracyline, Ceftaziamie, Gentamicin, Nalidixic acid. All discs were purchased from Hi-Media, India.

2.5. Media used for the assay

The Muller Hinton Agar was prepared and sterilized at 121°C and used to inoculate the isolates then incubated at 37°C for 24 hrs.

2.6. Insect immunization and haemolymph collection

Insect immunization was performed by injecting final instar larvae of *Diplocodes trivialis* with 10 µl of approximately 1×10^6 (cells/ml) viable, log phase bacteria dissolved in membrane-filtered saline using a thin-needled micro syringe. Bacterial strains were used for immunization separately or in combinations. Haemolymph was sampled add containing few crystals of phenylthiourea to prevent melanization. Usually, 10 ml of haemolymph was collected, from about 15 to 20 larvae (irrespective of sex), by piercing abdomen with a fine, sterile needle. Haemolymph was centrifuged for 15 min at 1,800 rpm, then the cell-free haemolymph was aliquoted (100 µl to 1 ml each), and stored at -20 °C for a week until investigated. The same procedures were applied to the control group except it was injected with saline without bacteria.

2.7. Antibacterial activity haemolymph

Hemolymph antibacterial assay was carried out using agar diffusion method. The 20 ml melted Luria agar media (LA. pH:7) was placed in the petri dishes (100 mm diameter), then the medium surface was impregnated with the 24-hour-grown strains (1.5×10^6 cells per ml). Twenty microliter hemolymph was poured in the wells of the plate and then the media culture was incubated at 37°C for 24 hours. The formation of inhibition zone (the lack of growth) represented the antibacterial hemolymph effect in the medium. Antibacterial assay was conducted on resistant bacteria with number of antibiotics resistance and virulence factors producing isolates.

2.8. Statistical analysis

Statistical Package for the Social sciences (Spss,2003) was used to analyse the data using descriptive analysis such as mean, standard deviation, frequencies, percentage, chisquare, and one way ANOVA and presented using Tables, Bar, and Pie charts.

3. RESULTS

The antibiotic resistance of *S.typhimurium* in different meat samples against the selected antibiotics was shown in Table 1. and Fig.1. It revealed that GS₇ isolate of goat meat was found to be highly resistant (70 %) towards majority of the antibiotics. The zone of inhibition was 8, 13, 12, 14, 11, and 10 mm against Cotrimaxazole, Centriaxone, Nitrofurantoin, Ciprofloxain, Chloramphenicol, and Ampicillin respectively. The next resistant isolate was CS₁ of chicken meat sample (60 %), it was resistant against Cotrimoxazole (20mm), Nitrofurantoin (11mm), Ciprofloxain (NI), Chloramphenicol (12mm), Ampicillin (NI), Tetracycline (NI) and Gentamicin (12 mm). Most of the *S.typhimurium* isolates (CS₂, CS₅, GS₆, FS₈, FS₉, CS₁₀ and FS₁₁) were found to be resistant against five antibiotics and found to be statistically significant. The least resistant sensitive isolates were GS₃ and FS₄ (30%). In general *S.typhimurium* was sensitive to Cotrimaxazole and Gentamicin subsequently *S.typhimurium* was highly resistant to Centriaxone, Nitrofurantoin, Ciprofloxain, Chloramphenicol, Ampicillin and Tetracycline. Haemolymph was more or less equally effective to the effect of antibiotics but haemolymph was moderately effective against *S.typhimurium* and inhibit the growth of *S.typhimurium*.

Table 1. Antibacterial activity of various antibiotics and larval haemolymph of *D.trivialis* against *S.typhimurium*

S. No.	Isolates name	Zone of Inhibition (mm)											
		COT	CTR	NIT	CIP	C	AMP	TE	CAZ	GEN	NA	%	HL
1.	CS ₁	20 _s	19 _i	11 _r	NI _r	12 _r	NI _r	NI _r	15 _i	12 _r	15 _s	60	18
2.	CS ₂	23 _s	13 _r	09 _r	18 _i	13 _i	NI _r	09 _r	16 _i	08 _r	24 _i	50	13
3.	GS ₃	23 _s	20 _i	NI _r	20 _s	14 _i	NI _r	NI _r	16 _i	16 _s	20 _s	23	17
4.	FS ₄	20 _s	20 _i	NI _r	20 _s	11 _r	NI _r	19 _s	19 _s	15 _s	12 _s	30	17
5.	CS ₅	20 _s	22 _s	17 _s	13 _r	11 _r	12 _r	12 _r	20 _s	17 _s	12 _r	50	NI
6.	GS ₆	22 _s	13 _r	09 _r	18 _i	14 _i	NI _r	NI _r	16 _i	12 _r	15 _i	50	15
7.	GS ₇	08 _r	13 _r	12 _r	14 _r	11 _r	NI _r	10 _r	20 _s	20 _s	24 _s	70	NI
8.	FS ₈	21 _s	23 _s	18 _s	24 _s	10 _r	NI _r	12 _r	10 _r	16 _s	12 _r	50	18
9.	FS ₉	23 _s	11 _r	12 _r	18 _i	14 _i	NI _r	12 _r	16 _i	10 _r	15 _i	50	18
10.	CS ₁₀	08 _r	12 _r	10 _r	12 _r	15 _i	18 _s	18 _s	20 _s	18 _s	NI _r	50	18
11.	FS ₁₁	09 _r	10 _r	12 _r	14 _r	14 _i	20 _s	22 _s	20 _s	18 _s	12 _r	50	15

Mean ± S.D	17.91±6.27	16.00±4.80	10.00±5.73	15.55±6.28	12.64±1.69	4.55±8.00	10.36±7.75	17.09±3.11	14.73±3.74	14.64±6.68	13.55±6.89
χ^2 value	17.875*	29.333*	30.250*	31.167*	18.562*	11.344*	29.333*	13.750*	31.625*	15.812*	
ANOVA	0.900*	5.454*	0.308#	0.016#	0.366#	0.318#	0.798#	2.323#	1.061#	2.565#	

COT - Cotrimoxazole, CTR - Centriaxone, NIT - Nitrofurantoin, CIP - Ciprofloxian, C - Chloramphenicol, AMP - Ampicillin, TE - Tetracycline, CAZ - Ceftazidime,

GEN -Gentamicin, NA - Nalidixic acid, HL-Haemolymph,NI-No inhibition.

S - Sensitive, R - Resistance, I - Intermediate * - Significant at $\chi^2_{0.05}$; ♦ - Not significant * - Significant at 0.05 (F); # - Not significant

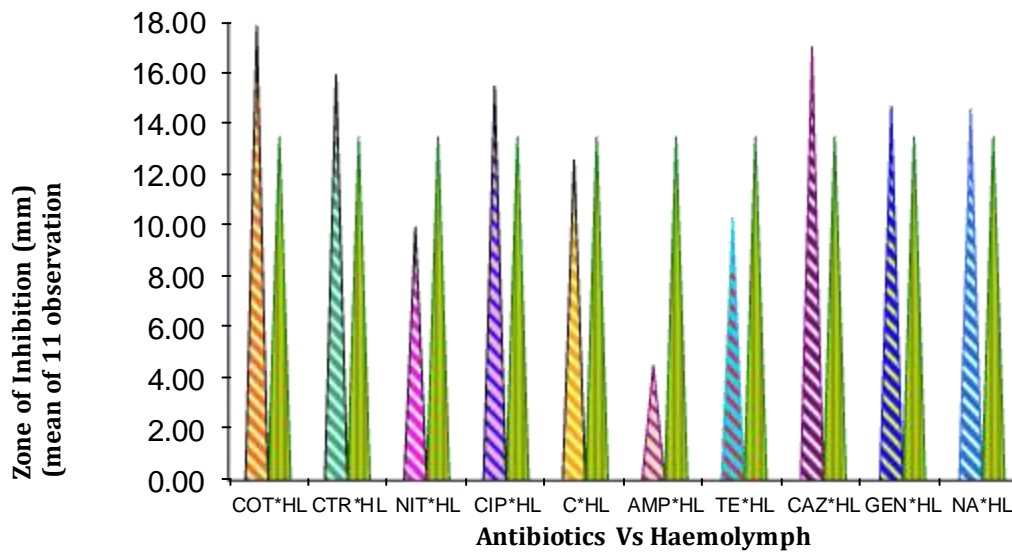


Chart -1: Antibacterial activity of various antibiotics and larval haemolymph of *D.trivialis* against *S.typhimurium*

Chart.2 the antibiotic resistance of *E.coli* in fish meat was the maximum (90 %) in FE₁₂ and FE₁₃ isolates. The isolates were resistant to most of the antibiotics viz Centriaxone, Ciprofloxain, Chloramphenicol, Ampicillin, and Tetracycline. All the *E.coli* isolates were sensitive to Nalidixic acid except GE₅ and all were highly resistant to Ampicillin. When compared to goat meat and fish meat, the chicken meat isolates were found to be least resistant to antibiotics. The antibiotic effect of haemolymph was equal to the effect of antibiotics. Haemolymph effectively kills the *E.coli* and found to be statistically significant.

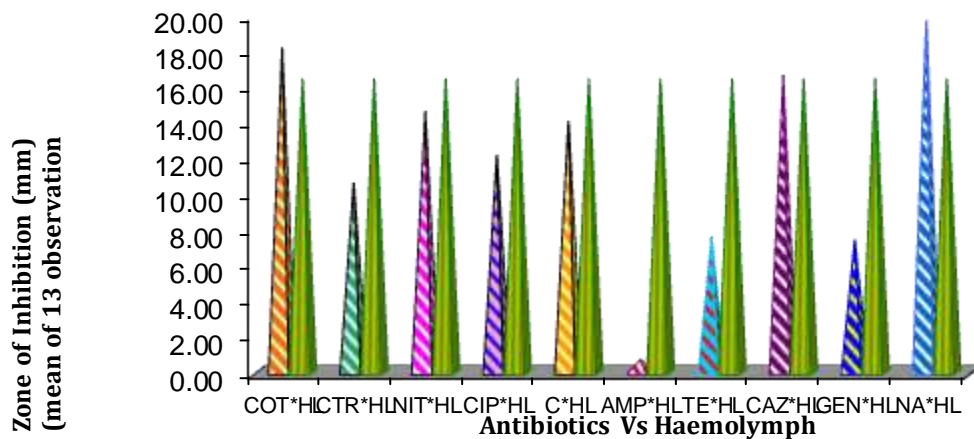


Chart -2: Antibacterial activity of various antibiotics and larval haemolymph of *D.trivialis* against *E.coli*

Table 3 and charts reflected the antibiotic resistance of *C.jejuni* which was observed only in few isolates GCa₁, CCa₂ and CCa₃ and not in fish meat sample. *C.jejuni* showed moderate resistance towards antibiotics. The only goat meat isolate (GCa₁) was resistant against Co-trimoxazole, Centriaxone, Nitrofurantoin, Ciprofloxain, and Chloramphenicol but it was sensitive to Ampicillin, Tetracycline, Ceftazidime, Gentamicin and Nalidixic acid. The other two isolates of chicken meat (CCa₂ and CCa₃) were sensitive to Co-trimoxazole, Centriaxone, Nitrofurantoin, Ciprofloxain, Ampicillin and Gentamicin but it was resistant to Chloramphenicol, Tetracycline, Ceftazidime and Nalidixic acid. The pathogen of chicken meat was found to be comparatively sensitive than the goat meat pathogens. Haemolymph inhibited the growth of *C.jejuni* effectively when compared to antibiotics. It was highly effective no Ampicillin, Gentamicin and Ceferiaxon on effects.

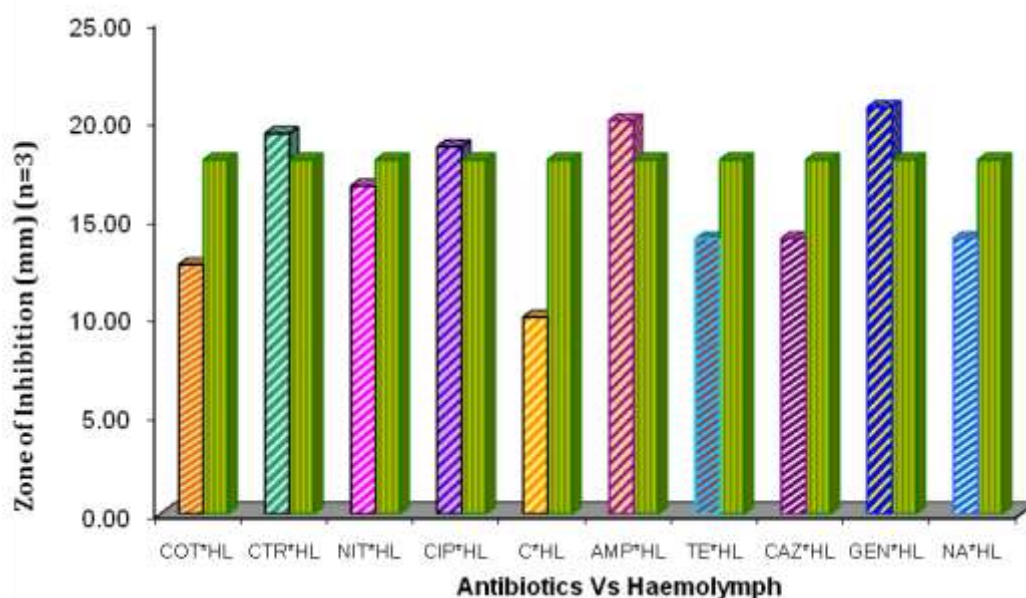


Chart. 3. Antibacterial activity of various antibiotics and larval haemolymph of *D.trivialis* against *C.jejuni*

4. DISCUSSION

The antimicrobial proteins present in insects, may be a good choice to replace bacteria resistant chemical compounds. Since these insects are normally present in all habitats, they are exposed to many invasive and harmful microbes in the nature. In the past century, researches concluded that insect immune system is able to cope with invading microbes (Seraj *et al.*, 2003; Boman *et al.*, 1993 and Hoffman *et al.*, 1993). So far, about 50 antibacterial molecules are isolated from the insects by stimulating their immune system. (Hultmark, 1993). Insect haemolymph was known to provoke the immune response. Hence several studies have been conducted in this area. By stimulating the immune system of bee's (*Apis spp.*) haemolymph (Kitel *et al.*, 2013), sand fly's (*Phlebotomus duboscqi*) parasite-immunized haemolymph (Boulanger, *et al.*, 2004), the greater wax moth's (*Galleria mellonella*) *P.aeruginosa*-injected haemolymph (Andrejko *et al.*, 2011) american cockroach's fungus injected haemolymph (James, 2012), the antibacterial activity was studied with the reports on apidaecian, defensin, cecropin, lectin like peptides which had the inhibitory effect on susceptible bacterial pathogens. It has been observed that in various insect species, when bacteria (*E.coli*) injected into the haemocoel elicit the synthesis of number of peptides and proteins which are active against the pathogenic bacteria and are secreted into the haemolymph (Gillespie *et al.*, 1997). In the present work, some features of humoral immune response of *Diplocodes trivialis* are reported. The presence of antibacterial activity against both the gram positive and gram negative bacteria in the haemolymph of *Dilpocodes trivialis* larvae was observed. These data are in agreement with the study made by Nimm *et al.*, (1997) reporting the presence of antibacterial activity in the haemolymph of sand flies *Lulzomyia longipalpis* after bacterial challenge. Nevertheless in our study we observed the presence of antibacterial activity in laboratory reared dragonfly larvae after experimental immunization with microbes.

CONCLUSION

The main idea of research is to study the antimicrobial activity against various bacterial isolates from different raw meat samples. From the present study it was concluded that three bacterial isolates. viz., *S.typhimurium*, *E.coli*, and *C.jejuni* were isolated from the raw chicken meat, goat meat, and fish meat. From this research it was found that *Diplocodes trivialis* has the ability to produce proteins to inhibit the growth of food borne pathogens in meat. Further work has to be done to improve the purification step so that the peptides can act higher than that of conventional antibiotics.

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BIOGRAPHIES



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