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Changes in Protein Profile of Bacterial-Challenged *Culex pipiens* Larvae

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Abstract: ***The present work aims to study the changes in protein profile of bacterial-challenged fourth instar Culex pipiens larvae. For achievement this research, fourth larval instars of Culex pipiens were challenged with Gram (+) bacteria, Staphylococcus aureus and gram (-) bacteria, Klebsiella pneumoniae and mix of the two bacterial strains to study the effect of bacteria on Culex pipiens larvae protein profile quantitative and qualitative at 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 hours postinfection (h.p.i.). The results showed a significant increase of total protein of the bacterial-challenged fourth instar larvae and then declined over time. Significant gradual increases in total soluble protein concentration were observed within each treatment group at all tested times. Such increases reached plateau phase at 36 h.p.i. Significant increase in total soluble protein was observed in the case of treated larvae as compared with controls. Whole body proteins of control and bacterial-challenged fourth instar larvae were separated using native and SDS-PAGE at different times postinfection. The results of native-PAGE demonstrated that there were differences in the overall protein banding pattern in the infected larvae as compared to control. The results of the SDS-PAGE clarified that the molecular weight of the separated proteins ranged from 140 to 14 KDa and bacterial infection had led to the induction of different proteins as compared to control.***

Keywords: protein profile, *Culex pipiens*, bacterial-challenged larvae

1. Introduction

Insects represent one of the most successful groups of evolution within the animal kingdom (about 75% of all animal species) in terms of species number Insects are exposed to a wide range of microbes throughout their life cycle and some species feed on animal or human blood infected with microorganisms and parasites. The amazing diversity and evolutionary success argue for an effective system of defense against infections. During evolution, insects developed a complex and effective innate immune system, which apparently differs from the adaptive immune system of vertebrates. However, there is no evidence for clonal selection mechanisms in insects and their immune system that shows no memory, their defense mechanisms are rapid, lasting up to a few days, and offering a particularly powerful resistance to microbial infections **(Royet, 2004; Ratcliffe *et al.*, 2011 and Vilcinskas, 2013).**

Mosquitoes significantly contribute to insect biodiversity and biomass, representing around 3500 described species, a few hundreds of which pose serious medical and economical risk **(Fang, 2010).** Mosquitoes are unquestionally the most important arthropod vectors of disease. The maintance and transmission of pathogens that cause malaria, lymphatic filariasis and numerous viral infections are absolutely dependent on the availability of competent mosquito vectors **(Youdeowei and Service, 1983).**

Concerning bacterial treatment effect on the protein profile of many insects, **Assar (2004a)** found that the bacterial infection reduce the protein content in the larvae of *Musca domestica*, while **Assar (2004b)** proved that the total protein content of the flesh fly, *Parasarcophaga aegyptiaca* increased after treatment with bacteria. Furthermore, **El Sobky *et al.*, (2006)** reported that the protein content of second instar larvae of *Culex pipiens* decreased after bacterial treatment. **Abo El-Mahasen (2007a)** worked on the lepidopteran *Spodoptera littoralis* larvae infected by *Bacillus thuringiensis* and cause decrease in the total protein content . Amarked decrease in the total protein content in the whole body homogenate of *Culex pipiens* larvae treated with *B. thuringiensis* **(Abo El-Mahasen, 2007b)**. **Chalk *et al.*, (1994)** studied the effect of bacteria on the protein pattern as well as following bacterial inoculation, a basic, inducible antibacterial peptide has been detected using native PAGE at pH 4, which corresponds with a 4.5 kDa peptide detected by tricine SDS-PAGE followed by silver staining. Here we study the effect of gram (+) and gram (-) bacteria on *Culex pipiens* larvae protein content quantitative and qualitative (native and SDS-PAGE).

2. Materials and Methods

2.1 Insects and Bacterial Strains

Mosquito samples were obtained from breeding habitat in Giza Governorate, Egypt. Mosquito larvae reared and colonized in the in sectary of the Department of zoology, Faculty of Science, South Valley University. Adults were identified morphologicaly using taxonomic keys of **Harbach (1985).** The stock colony of the adult mosquitoes was maintained under laboratory conditions (27 $\pm$ 2 °C and 60-70% RH) for supplying clean adults of known ages, according to the method described by **Adham *et al.*, (2003).**

Gram (+) bacteria, *Staphylococcus aureus* and gram (-) bacteria, *Klebsiella pneumoniae* were obtained from the Unit of 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%

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NaCl, at 37 °C in a rotary shaker. Bacterial challenge was performed by feeding newly moulted fourth instar larvae of *Cx. pipiens* with diluted bacteria. Bacterial strains were used for immunization separately and in combinations.

1- Whole Body Collection:

Cx. pipiens fourth instar larvae were kept without food for 6 hrs then they classified into four groups . The first group was control (**C**) (normal larvae without any infection). The second group was *K. pneumoniae* ($\mathbf{T_k}$) (infected larvae with gram - bacteria, *K. pneumoniae* for 24 hrs). The third group was *S. aureus* ($\mathbf{T_s}$) (infected larvae with gram + bacteria, *S. aureus* for 24 hrs). Finally, the fourth group was Mix ($\mathbf{T_m}$) (infected larvae with both gram - bacteria, *K. pneumoniae* and gram + bacteria, *S. aureus* for 24 hrs). Both control and bacterial-challenged fourth instar larvae were collected after 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72 h.p.i. at 4°C. A bout 1 µl Phenyl Methyl Sulfonyl Fluoride (PMSF) were added to the collected sample to prevent protein degradation and stored at -80°C until it used.

2- Quantitative Protein Analysis:

The total protein content of the whole body was quantified spectrophotometrically in both the control and each bacterial challenged larvae group samples at 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h.p.i. according to the method described by **Bradford (1976)**. This method is based on the observation that Coomassie Brilliant Blue G-250 exists in two different colour forms, red and blue. The red colour is converted to the blue colour upon binding of the dye to protein. This binding causes a shift in maximum absorption of the dye from 465 to 595 nm. The intensity of the colour was measured at 595 nm.

3- Native Polyacrylamide Gel Electrophoresis (PAGE):

Native-PAGE of both control and each bacterial-challenged fourth instar larvae groups at 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h.p.i. was carried out in the different whole body samples. It was carried out using 15% polyacrylamide gels pH 4, using a discontinous buffer system accordding to **Gabriel, 1971**. The acrylamide/ bisacrylamide ratio was 60: 0.8. The gels were run at 200 V until the tracker dye (Bromphenol blue) was running off the gel (approximately 2.5 h).

4- SDS Polyacrylamide Gel Electrophoresis (PAGE):

Sodium dodecylsulfate (SDS-PAGE) of control and each bacterial-challenged fourth instar larvae groups at 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h.p.i. was carried out. SDS-PAGE of denatured proteins was carried out using 15% polyacrylamide gels pH 8.8, in a discontinous buffer (**Maizel and Jr, 1971**). The acrylamide/ bisacrylamide ratio was 50: 1. The gels contained no SDS before electrophoresis. The protein samples were pretreated with 1% SDS and 1% β-mercaptoethanol for 5-10 min. at 100 °C. The gels were run at 150 V until the tracker dye (bromphenol blue) was leaving the gel (approximately 2 h.). All gels were fixed in 20% 5 sulfosalicylic acid, stained with Coomassie Brilliant Blue R250 and destained in 7% acetic acid.

3. Results

Quantitative protein analysis:

As shown in Table 1 and Fig. 1, quantitative protein analysis of the whole body homogenate of control and bacterial-challenged fourth instar larvae of *cx. pipiens* was estimated at collected 6 , 12 , 18 , 24 , 30 , 36 , 42 , 48 , 54 , 60 , 66 , and 72 h.p.i. Generally, significant gradual increases in total soluble protein concentration were observed within each treatment group at all tested times. Such increase reached plateau phase at 36 h.p.i. (Fig. 1). Significant increase in total soluble protein was observed in the case of treated larvae at 18 and 48 h.p.i. with *K. pneumoniae* and in the case of treated larvae at 18 h.p.i. with *S. aureus* also in the case of treated larvae at 18 and 24 h.p.i. with mixed infection as compared with controls ($P<0.01$). Mixed infection exhibited significantly lower protein concentration than separate bacterial infection and control, as well ($P<0.01$), over the first 6 h.p.i.

Firstly, total protein concentration of *K. pneumoniae* -infected samples decreased after 6 h.p.i. Over time, total protein concentration increased untile after 48 h.p.i. (12, 18, 24, 36, 42 and 48 h.p.i.) (Fig. 1) with exception in the case of 30 h.p.i. as the total protein concentration decreased. Finally, significant decrease in total protein concentration was observed in the last four times (54, 60, 66 and 72 h.p.i.) as compared with control ($P<0.01$).

Secondly, total protein concentration of *S. aureus* -infected samples increased with time untile after 48 h.p.i. (6, 12, 18, 24, 36, 42 and 48 h.p.i.) (Fig. 1) with exception in the case of 30 h.p.i. as the total protein concentration decreased. Lastly, significant decrease in total protein concentration was observed in the last four times (54, 60, 66 and 72 h.p.i.). as compared with control ($P<0.01$).

Thirdly, total protein concentration of Mix-infected samples decreased after 6 h.p.i. With time, total protein concentration increased untile after 48 h.p.i. (12, 18, 24, 42 and 48 h.p.i.) (Fig. 1) with exception in the case of 30 and 36 h.p.i. as the total protein concentration decreased. Finally, significant decrease in total protein concentration was observed in the last four times (54, 60, 66 and 72 h.p.i.) in comparison with control ($P<0.01$).

One way analysis of variance revealed over all significant differences in total protein concentration between control and treated samples. Post hoc tests revealed significant differences between control and all treated samples at all tested times ($P<0.01$).

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Table 1: Quantitative protein analysis of the bacterial-challenged whole body of fourth instar larvae of *Cx. pipiens* at different times postinfection

	Protein concentration (mg/g)					
	C	T_k	T_s	T_m	F	P
6	20.89±0.17	15.97±0.08*	23.87±0.04*	17.16±0.06*	1298.749	P<0.01
12	20.89±0.17	22.85±0.02*	27.30±0.03*	25.65±0.03*	1081.796	P<0.01
18	20.93±0.06	28.59±0.02*	32.67±0.03*	27.11±0.06*	11418.939	P<0.01
24	20.93±0.06	26.96±0.06*	23.15±0.02*	26.92±0.02*	4286.051	P<0.01
30	20.95±0.10	19.50±0.02*	17.55±0.06*	15.22±0.09*	1140.448	P<0.01
36	20.95±0.10	21.32±0.03*	21.77±0.02*	19.98±0.07*	143.865	P<0.01
42	21.03±0.15	23.64±0.08*	22.36±0.04*	23.94±0.02*	224.367	P<0.01
48	21.03±0.15	28.09±0.05*	28.43±0.04*	24.88±0.07*	1476.483	P<0.01
54	21.93±0.06	19.38±0.09*	20.24±0.09*	18.05±0.03*	534.636	P<0.01
60	21.93±0.06	15.72±0.02*	19.95±0.05*	16.42±0.01*	5291.244	P<0.01
66	20.89±0.17	16.93±0.05*	17.42±0.11*	16.94±0.05*	324.391	P<0.01
72	20.89±0.17	17.77±0.04*	20.08±0.02*	19.14±0.02*	234.303	P<0.01

*. The mean difference is significant at the 0.01 level

2- Native and SDS PAGE:

Whole body proteins of control and bacterial-challenged fourth instar larvae were separated using native and SDS-PAGE at 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h.p.i. The results of native-PAGE demonstrated differences in the overall protein banding pattern in the infected larvae as compared to control. The results of the SDS-PAGE clarified that the molecular weight of the separated proteins ranged from 140 to 14 KDa and bacterial infection had led to the induction of different proteins as compared to control.

Results represented in Fig. (2) shows the native PAGE of different studied groups, control (C) (normal larvae without any infection), *K. pneumoniae* (**T_k**) (infected larvae with gram - bacteria, *K. pneumoniae*) and *S. aureus* (**T_s**) (infected larvae with gram + bacteria, *S. aureus*) and Mix (**T_m**) (infected larvae with both gram - bacteria, *K. pneumoniae* and gram + bacteria, *S. aureus*) which electrophoresed after 6, 12, 18, and 24 h.p.i., While Fig. (3) shows native PAGE of different studied groups **(C, T_k, T_s and T_m)** electrophoresing after 30, 36, 42, and 48 h.p.i. Finally, Fig. (4) shows native PAGE of different studied groups **(C, T_k, T_s and T_m)** which electrophoresed after 54, 60, 66, and 72 h.p.i. as they separated into different number of bands (Table 2).

Results registered in Fig. (5) shows the SDS-PAGE of different studied groups, control (C) (normal larvae without any infection), *K. pneumoniae* (**T_k**) (infected larvae with gram - bacteria, *K. pneumoniae*) and *S. aureus* (**T_s**) (infected larvae with gram + bacteria, *S. aureus*) and Mix (**T_m**) (infected larvae with both gram - bacteria, *K. pneumoniae* and gram + bacteria, *S. aureus*) which electrophoresed after 6, 12, 18, and 24 h.p.i. While Fig. (6) shows SDS-PAGE of different studied groups **(C, T_k, T_s and T_m)** which electrophoresed after 30, 36, 42, and 48 h.p.i. At last, Fig. (7) shows SDS-PAGE of different studied groups **(C, T_k, T_s and T_m)** that electrophoresed after 54, 60, 66, and 72 h.p.i. as they separated into different number of bands with molecular weight ranged from 140 to 14 KDa (Table 3).

Table 2: Total number of bands separated by native PAGE of the bacterial-challenged whole body of fourth instar larvae of *Cx. pipiens* at different times postinfection.

h. p.i.	Total number of bands C = 10 bands		
	T_k	T_s	T_m
6	9	12	12
12	12	11	12
18	13	12	12
24	11	12	12
30	11	12	11
36	12	13	13
42	9	9	9
48	9	9	7
54	10	11	11
60	8	9	7
66	9	12	12
72	13	11	11

Table 3: Total number of bands separated by SDS-PAGE of the bacterial-challenged whole body of fourth instar larvae of *Cx. pipiens* at different times postinfection.

h. p.i.	**Total number of bands C = 9 bands**		
	T_k	**T_s**	**T_m**
6	9	9	8
12	9	11	12
18	10	9	11
24	9	11	12
30	8	12	9
36	9	12	12
42	10	10	13
48	9	11	13
54	10	14	13
60	10	13	11
66	12	13	13
72	10	9	7

4. Discussion

The main objective of the current work is to study the changes in protein profile of bacterial-challenged fourth instar *Culex pipiens* larvae. Quantitative protein analysis of the whole body of control and bacterial-challenged fourth instar larvae of *Cx. pipiens* was estimated at different hours postinfection. Significant increase in total soluble protein was observed in the case of treated larvae as compared with control. Agreeable results were reported by **Seufi *et al.*, 2009** who observed significant increase of the total protein in the bacterial-challenged *Spodoptera littoralis* larvae and then declined over time. In contrast to the present results,

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Abuldahab *et al.* (2011) noticed a significant decrease in the level of total soluble protein in the larvae of *M. domestica* treated with bacteria at different time intervals as compared to control, **El Sobky *et al.*, (2006)** stated that the protein content of second instar larvae of *Cx. pipiens* decreased after bacterial treatment and **Abo El-Mahasen (2007b)** demonstrated that a marked decrease in the total protein content in the whole body homogenate of *Cx. pipiens* larvae treated with *B. thuringiensis*.

Polyacrylamide gel electrophoresis (PAGE) has been extensively used as an excellent tool for the separation of proteins from all living organisms (**Zacharius *et al* 1969**). The vast majority of recent studies on insect proteins have used electrophoretic techniques. Polyacrylamide gel, with the advantages of high sensitivity and resolving power, is generally the most efficient medium (**Wyatt and Pan, 1978**).

Results obtained from the native-PAGE of whole body of bacterial-challenged larvae at different time intervals demonstrated that there were changes in the bulk protein content of the infected larvae as compared to control. Similarly, SDS-PAGE results showed characteristic protein bands appeared in *S. aureus*, *K. pneumoniae* and mix-challenged larvae with relatively low and high molecular weights at different times postinfection. Thus, the bacterial infection was capable of changing the profile of whole body proteins qualitatively. The appearance of different bands in treated larvae may be attributed to the induction of new proteins in the immune reactions. The synthesis of new immune proteins may be a result of simultaneous induction of challenged-bacteria (**Rasmuson and Boman, 1979**) and a fast rate of transcription (**Sun, 1992**).

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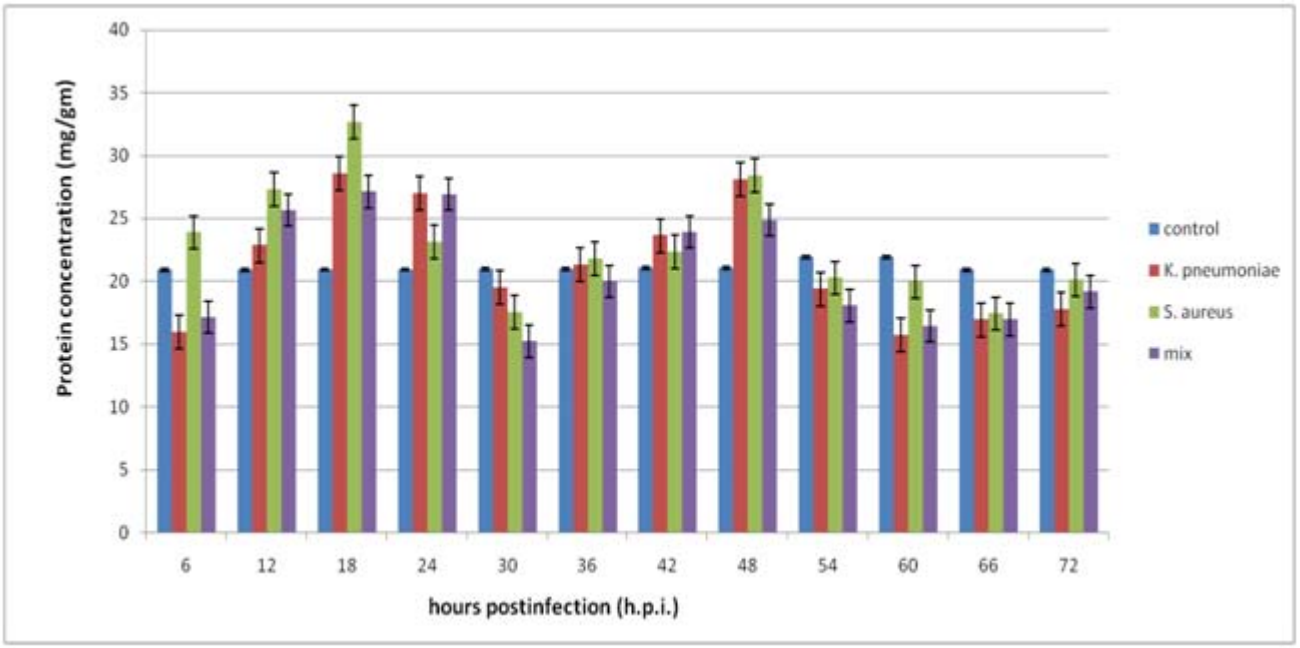


Figure 1: Quantitative protein analysis of the bacterial-challenged whole body of fourth instar larvae of *cx. pipiens* at different times postinfection.

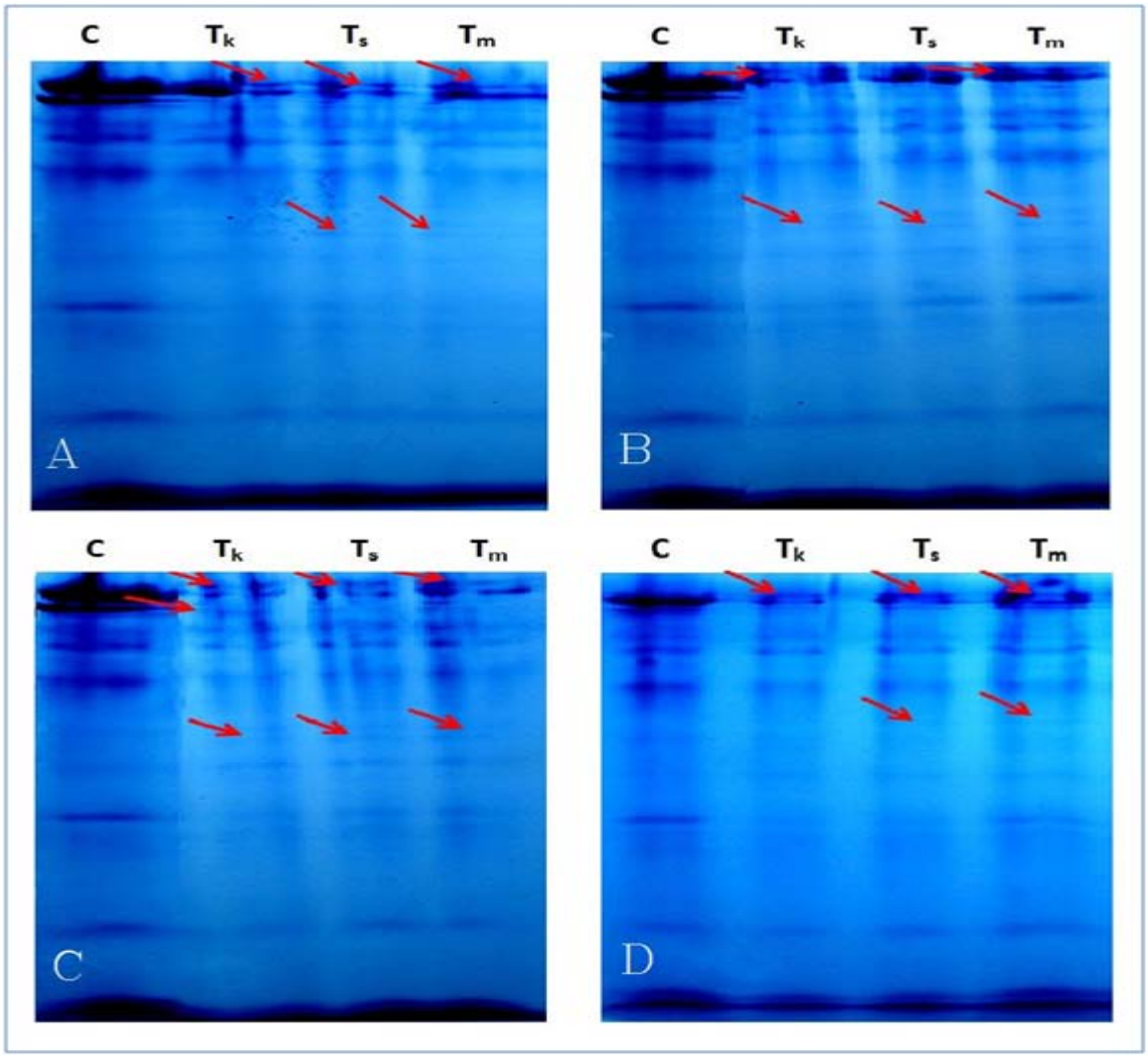


Figure 2: Native PAGE of different studied groups control (C) (normal larvae without any infection), *K. pneumoniae* ($\mathbf{T_k}$) (infected larvae with gram - bacteria, *K. pneumoniae*) and *S. aureus* ($\mathbf{T_s}$) (infected larvae with gram + bacteria, *S. aureus*) Mix ($\mathbf{T_m}$) (infected larvae with both gram - bacteria, *K. pneumoniae* and gram + bacteria, *S. aureus*). (A&B&C&D) the different studied group after 6, 12, 18, 24 h postinfection respectively.

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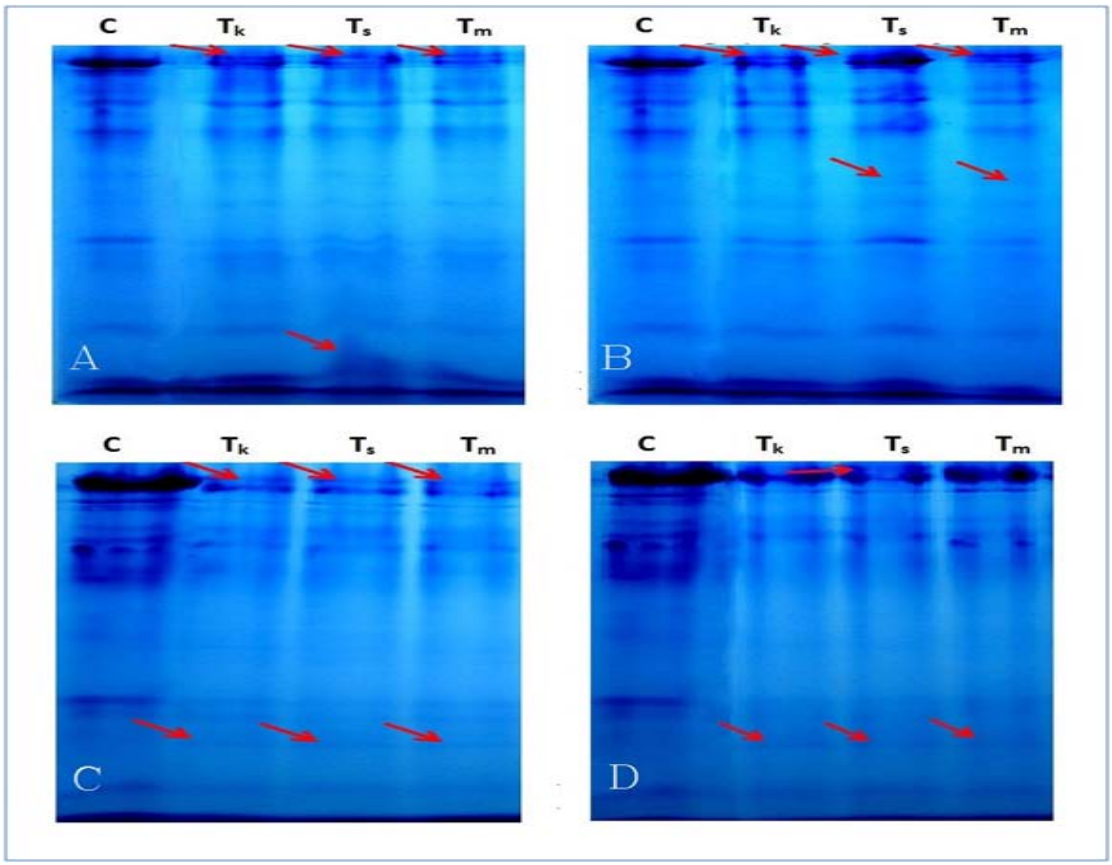


Figure 3: Native PAGE of different studied groups control (C) (normal larvae without any infection), *K. pneumoniae* ($\mathbf{T_k}$) (infected larvae with gram - bacteria, *K. pneumoniae*) and *S. aureus* ($\mathbf{T_s}$) (infected larvae with gram + bacteria, *S. aureus*) Mix ($\mathbf{T_m}$) (infected larvae with both gram - bacteria, *K. pneumoniae* and gram + bacteria, *S. aureus*). (A&B&C&D) the different studied group after 30, 36, 42, 48 h postinfection respectively.

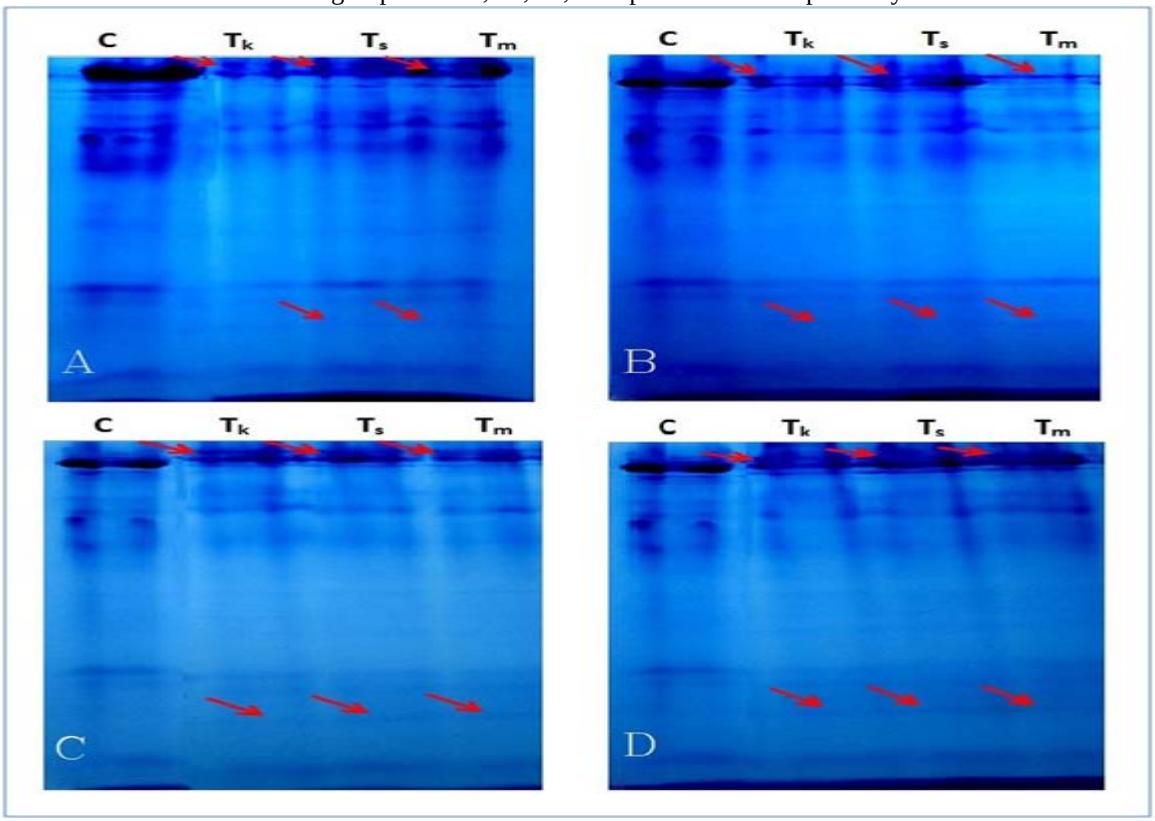


Figure 4: Native PAGE of different studied groups control (C) (normal larvae without any infection), *K. pneumoniae* ($\mathbf{T_k}$) (infected larvae with gram - bacteria, *K. pneumoniae*) and *S. aureus* ($\mathbf{T_s}$) (infected larvae with gram + bacteria, *S. aureus*) Mix ($\mathbf{T_m}$) (infected larvae with both gram - bacteria, *K. pneumoniae* and gram + bacteria, *S. aureus*). (A&B&C&D) the different studied group after 54, 60, 66, 72 h postinfection respectively.

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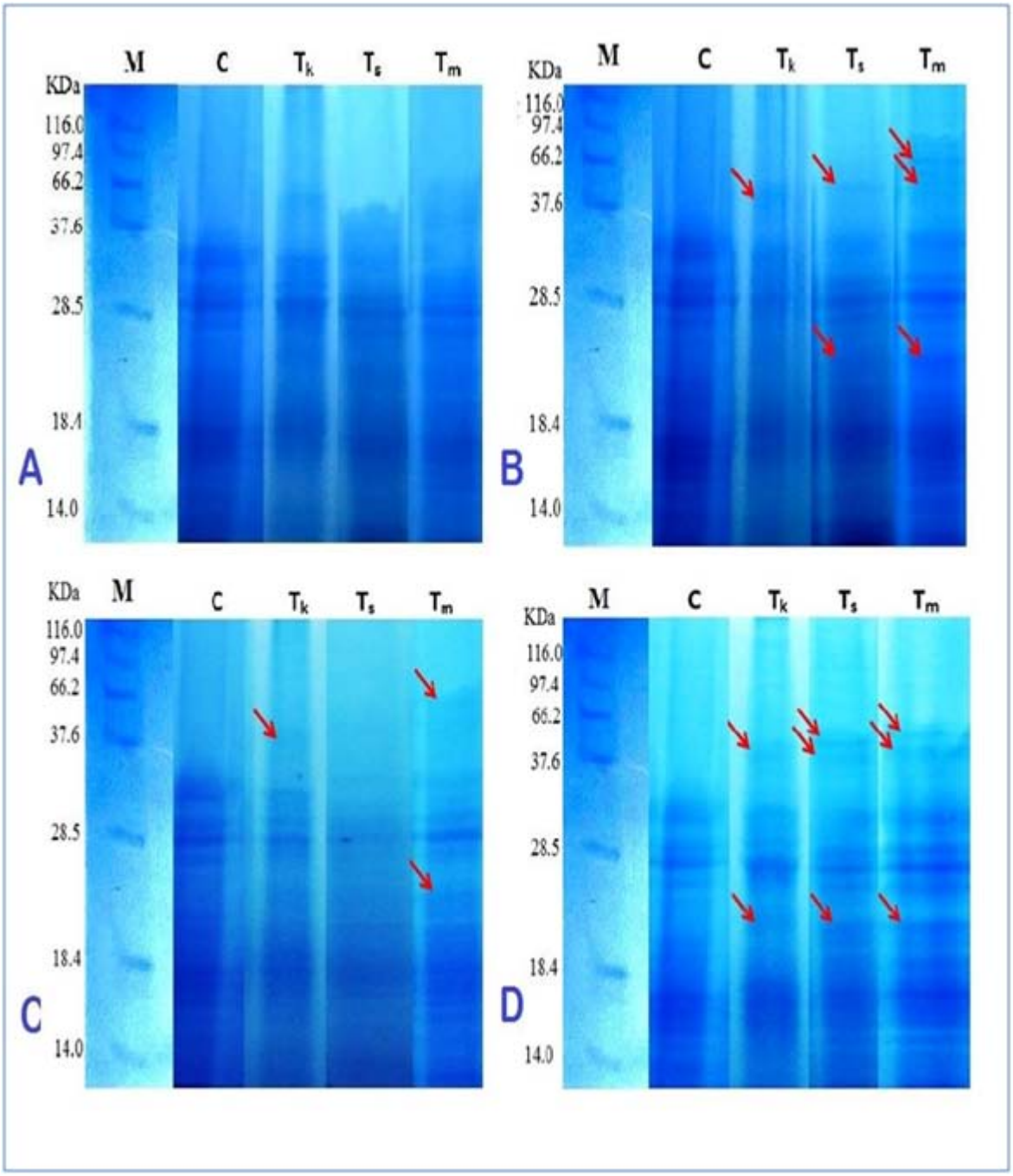


Figure 5: SDS PAGE of different studied groups control (C) (normal larvae without any infection), *K. pneumoniae* ($\mathbf{T_k}$) (infected larvae with gram - bacteria, *K. pneumoniae*) and *S. aureus* ($\mathbf{T_s}$) (infected larvae with gram + bacteria, *S. aureus*) Mix ($\mathbf{T_m}$) (infected larvae with both gram - bacteria, *K. pneumoniae* and gram + bacteria, *S. aureus*). (A&B&C&D) the different studied group after 6, 12, 18, 24 h postinfection respectively.

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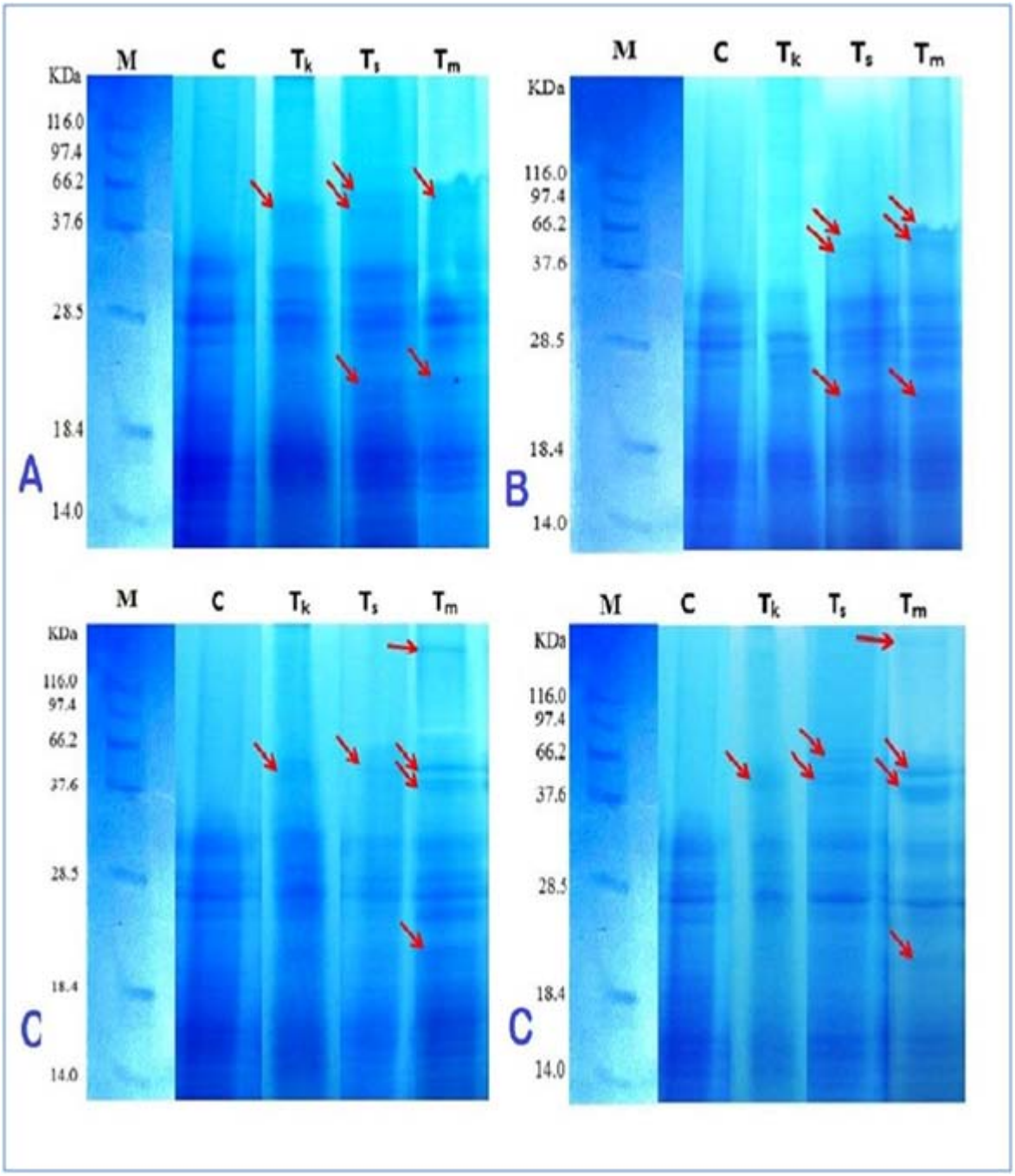


Figure 6: SDS PAGE of different studied groups control (C) (normal larvae without any infection), *K. pneumoniae* ($\mathbf{T_k}$) (infected larvae with gram - bacteria, *K. pneumoniae*) and *S. aureus* ($\mathbf{T_s}$) (infected larvae with gram + bacteria, *S. aureus*) Mix ($\mathbf{T_m}$) (infected larvae with both gram - bacteria, *K. pneumoniae* and gram + bacteria, *S. aureus*). (A&B&C&D) the different studied group after 30, 36, 42, 48 h postinfection respectively.

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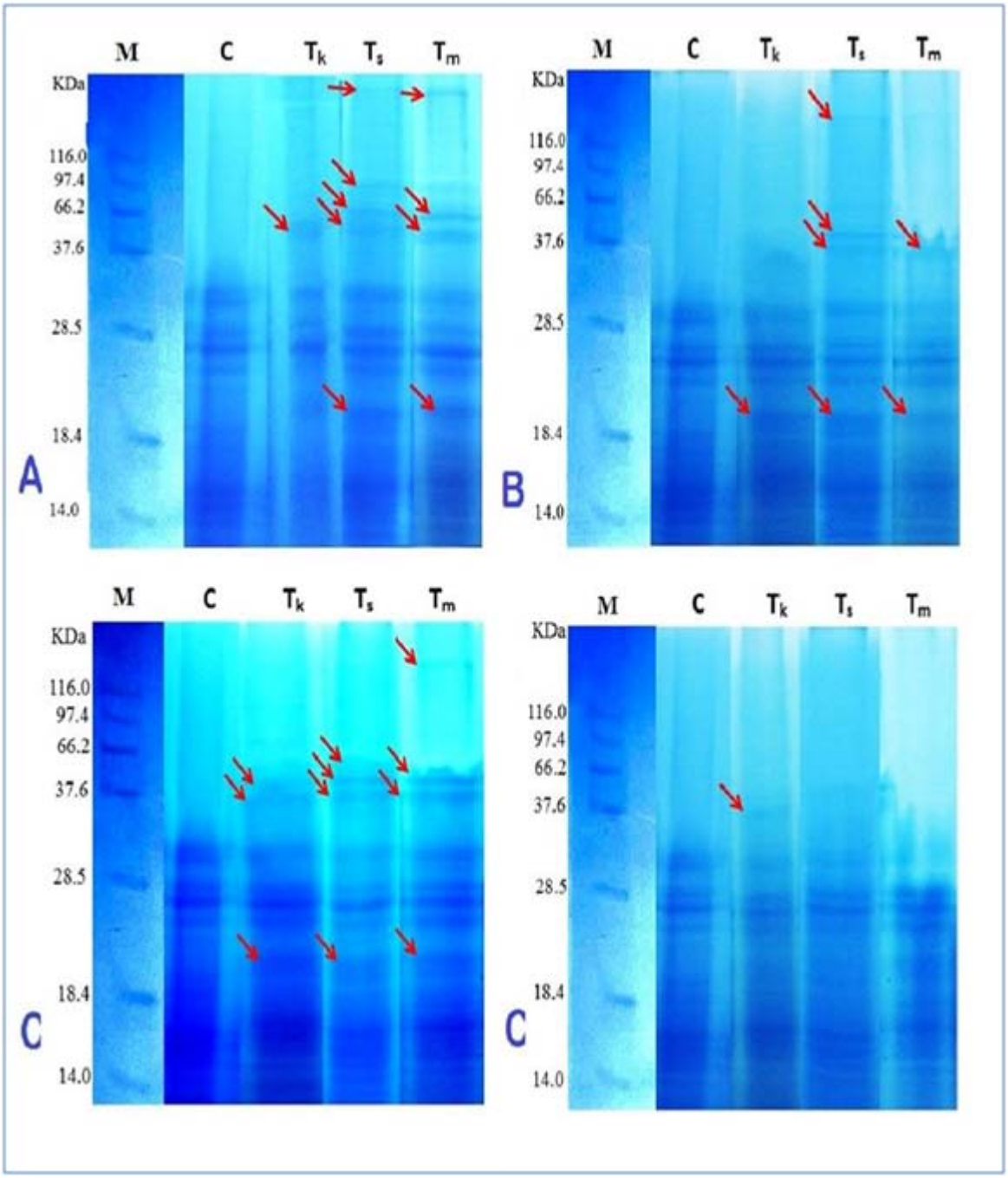


Figure 7: SDS PAGE of different studied groups control (C) (normal larvae without any infection), *K. pneumoniae* ($\mathbf{T_k}$) (infected larvae with gram - bacteria, *K. pneumoniae*) and *S. aureus* ($\mathbf{T_s}$) (infected larvae with gram + bacteria, *S. aureus*) Mix ($\mathbf{T_m}$) (infected larvae with both gram - bacteria, *K. pneumoniae* and gram + bacteria, *S. aureus*). (A&B&C&D) the different studied group after 54, 60, 66, 72 h postinfection respectively.

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