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Comparison of Midgut Hemagglutination Activity in Three Different Geographical Populations of *Anopheles stephensi*

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Abstract

Lectins that agglutinate red blood cells (RBCs) were demonstrated in *Anopheles stephensi* mosquito midgut extracts using human (four groups: A, B, AB and O, RH+) rat, sheep and rabbit blood cells. Only rabbit RBCs showed agglutination reaction against the midgut extracts. Significant differences in hemagglutinin titers and carbohydrate specifity were detected between male and female mosquitoes as well as among three different geographical populations of *Anopheles stephensi* from south of Iran. Overall agglutinin levels were increased following a blood meal. The highest hemagglintination titers were due to Kazerun population. All hemagglutination assays were versus rabbit RBCS. A significant difference was detected among the number of egg-float ridges. Iranshahr population was different from Bandar-abbas and Kazerun population in egg-float ridges number. Bandr-abbas population was in the intermediate category. Iranshahr population fell between mysoransis and intermediate group and Kazerun population was between intermediate and type form. This study presents the first report on the occurrence of heterogeneous anti Rabbit RBC agglutinins in the midget extracts of the different geographical populations of *An.stephensi* with the sugar – binding specificities. The sugar- inhibition pattern was different between & within geographical population of *An.stephensi.*

Keywords: Anopheles stephensi*, Midgut, Hemagglutinins, Geographical populations*

Introduction

Lectins in different insects are important for both the establishment of infection and parasites development in the gut and haemolymph (1-3). Insects display both cellular (haemocyte) and non-cellular (humeral) defense reactions to counteract diverse environmental pathogens (4, 5). Agglutinins are innate, naturally occurring molecules that participate in humeral immune mechanisms of insects and exhibit antibody-like roles (6). Many of the insect haemagglutinins are lectin or lectin-like molecules that are ubiquitous, non-enzymatic, carbohydrate-binding proteins or glycoproteins, and once bound to erythrocytes or other cells, usually cause their agglutination, and may also precipitate glycoconjugates (7). Many haematophagous diptera are vectors of pathogenic parasites of man and animals. Due to social and economic importance of the diseases caused by these organisms, some investigations have been focused on insect haemolymph or gut extract. Hemagglutinins involved in carbohydrate-binding specificities, because it is considered that they could exert an influence on host-parasite interactions in the appropriate vectors (7- 9).

It is suggested that interactions between parasites and vector gut walls may be mediated by the carbohydrates present on the surface of parasites and the lectins in the vector gut. Characteristic carbohydrate markers have been identified on the surface of parasites including T*rypanosome* and *Leishmania* (10, 11), and the midgut lectins have been identified from such vectors as *Rhodnius prolixus* (12) *Anopheles gambiae*(13), *Glossina austain* (14), *Phlebotomus papatasi* (15-19). In the present study, we

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identified a number of agglutination activities from different geographical populations of *Anopheles stephensi* from south of Iran, both sexes of the mosquitoes, and also blood-unfed and blood-fed females. Egg-float ridges of different geographical populations of *An. stephensi* were compared with those previously reported from India. *An. stephensi* is the main vector of human malaria in South Africa, and Middle East to Far East but there is little information on the occurrence of hemagglutinins in this mosquito.

Chen & Billingsley (20) reported a mannan-biuding lectin from the mosquitoes *Anopheles stephensi* (Liston) haemolymph. This paper reports, for the first time, the detection of hemagglutinins in the midgut extracts of male, female of *An. stphensi* and comprised midgut lectin activity of different geographical population of this species from south and southeast of Iran.

Materials and Methods

Source and maintenance of mosquitoes

Three geographical populations of An. stphensi from south and southeast of Iran including Bandar-Abbas, Kazerun & Iranshahr originated from a particular rural location were reared in insectaries and allowed to take blood on Guinea pigs three times a week, under a photoperiod of 12:12 light at 25-29°C and 80% relative humidity. Eggs were hatched in plastic treys containing water. Pupa were collected daily and placed in water inside a mosquito cage. Adults were maintained on 10% sucrose.

Counting Egg Float Ridges Number (EFRN)

Three geographical populations of *An. stephensi*, which had been originated from previously colonized from a particular rural location were used in order to investigate their EFRN. According to sample size calculation, sixty eggs of each colony from the insectarium population were selected randomly and the ridge number was counted by a microscope using ×10 and/or × 40 objectives.

Midgut dissection Adult males, unfed and fed females from each population were prechilled at-20°C for 10 min, and dissected in cold phosphate buffered saline (10 mM sodium phosphate, 150 mM NaCl, 25 mM KCl, pH 7.4). The technique of tissue isolation was similar to that reported by Russell et al (21). The midguts were washed three times with PBS and homogenized with a mechanical homogenizer and then spun down at 10000g for 10 minutes. The supernatants were kept at-20°C until used.

Hemagglutination assays using various RBCs

Different mammals red blood cells such as rabbit, rat, human (four blood groups A, B, AB, O RH positive) were used in haemagglutination tests.

Rabbit blood was collected in 10 ml tubes containing 1% heparin. Erythrocytes were washed three times by centrifugation at 800g for 15 min in cold 10 mM PBS, and resuspended at 2% concentration in PBS containing 2% BSA.

Protein assay Before setting the hemagglutination assay, we conducted the Bradford assay to estimate the protein concentration of each midgut extract.

Hemagglutination assays Hemagglutinaton assays were performed using 96 well V-bottomed microtitre plates. Double serial dilutions of gut extract, (each 2.5 µl in volume) were prepared with PBS in microtitre plates to give final dilution ranges of 2^{-1} to 2^{-10} prior to the addition of 2.5 µl of adjusted RBCs to each well. Then the plates were incubated for two hour, at room temperature.

Carbohydrate inhibition assay The sugar specificities of mosquito midgut lectins were investigated by competitive binding using the following sugars: D(+) galactose, D(+) mannose, L(-) fucose, lactose, N-acetyl-D-glucosamine, N-actyl-D-galactosomine and D(-) arabinose. Stock solutions of each carbohydrate were prepared at concentration of 300 mM in PBS pH 7.4. Two folded serial dilutions of gut extracts (each of 2.5 µl) were prepared in PBS followed by addition of 2.5 µl of appropriate

carbohydrate at the above initial concentration and left in RT for one hour.
The plates were incubated at room temperature for 120 min and then 2.5 µl of adjusted RBCs (rabbit blood cells) were added to the respective wells. The controls consisted of midgut extract, or carbohydrate plus RBSs, and PBS plus RBCs alone. Inhibitory sugars were recorded as those reducing agglutination by wells.

Results

Egg float ridges number (EFRN): With regard to Subbarao's categorizing method (22) Bandar-Abbas population (EFRN= 14, 15, 16) fell in intermediate group, Iranshar population (EFRN= 11, 12 13, 14, 15) in the Intermediate with a tendency to Mysoransis and Kazerun population (E.F.R.N= 13, 14, 15, 16, 17) in the Intermediate variant with a tendency to type form. (Fig 1- 4).

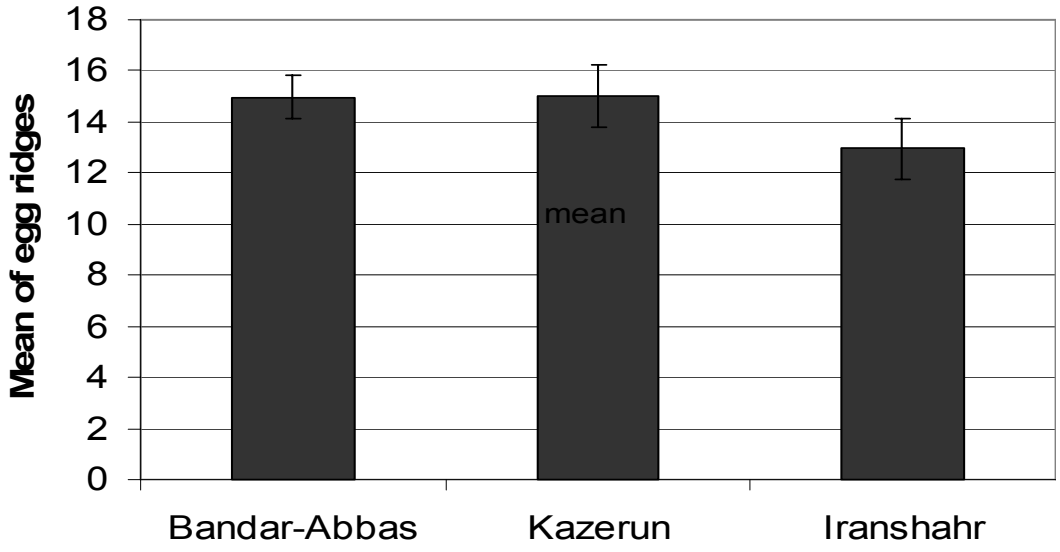

Fig. 1: Mean of egg ridges number of *Anopheles stephensi* from south of Iran (Bandar-Abbas, Kazerun and Iranshahr)

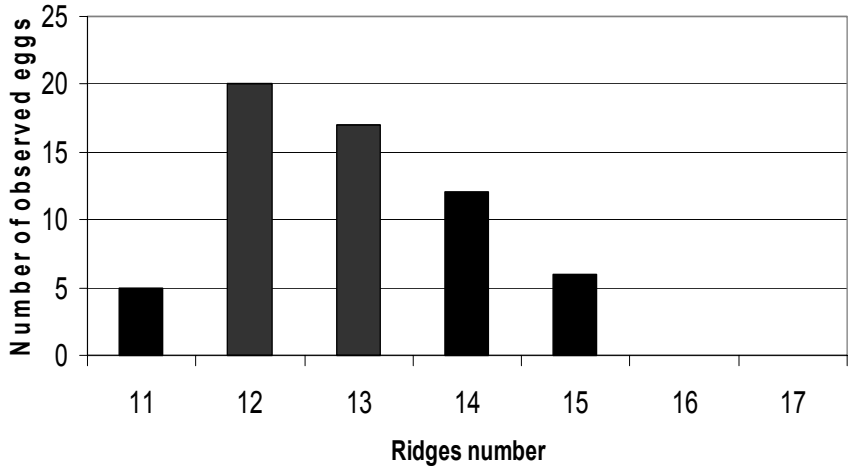

Fig. 2: Egg float ridges distribution of each population within Iranshahr population of *An. stephensi*

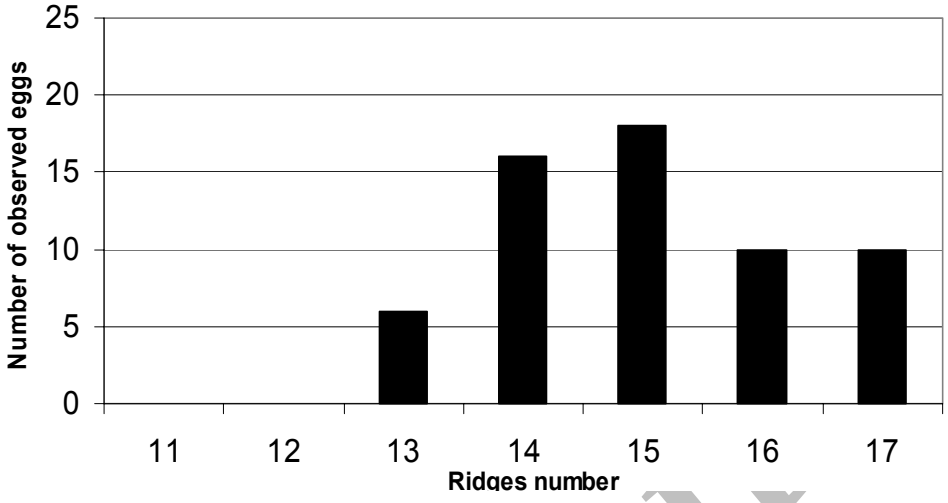

Fig. 3: Egg float ridges distribution of each population within Kazeron population of *An. stephensi*.

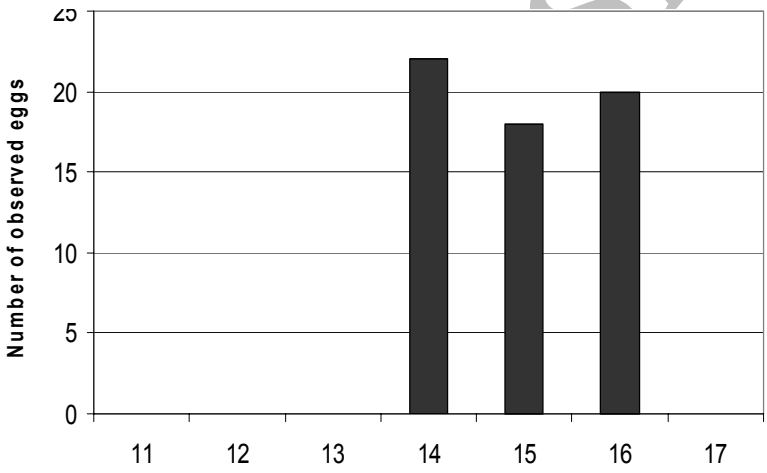

Fig. 4: Egg float ridges distribution of each population within Bandar-Abbass population of *An. stephensi*

Hemagglutination assays using various RBCs Midgut extracts of three geographical populations of *An. stephensi* from south and southeast of Iran (Iranshahr, Bandar-abbas & Kazerun) agglutinated Rabbit RBCs. Hemagglutination titers was varied regarding to sex, feeding and location of mosquitoes, ranged from 1:2 to 1: 64 (Table 1).

Table 1: Haemagglutination titers (figures in the parenthesis) of midgut extracts of three different geographical populations of *Anopheles stephensi* in south of Iran

Population	Midgut (male)	Midgut (blood unfed female)	Midgut (blood fed female)	BSA (control)	BS (control)
Kazerun	3* (8)	3 (8)	5-6 (32-64)	-	-
Iran-shahr	3 (8)	2 (4)	4 (16)	-	-
Bandar-abbas	1-2 (2-4)	2 (4)	3-4 (8-16)	-	-

The numbers in parentheses show endpoint titers expressed as the reciprocals of the dilutions (1/n)

* The number of wells that heamagglutination was seen

The highest midgut extract titers were found against rabbit RBCs in Kazerun population 1:8 in males, 1:8 in blood unfed females and 1: 64 in blood-fed females (Table 2).

In the three geographical populations hemagglutination activities were blood meal enhanced, and the hemagglutination titers were higher in blood-fed females than in sugar- fed females (Table 1).

Carbohydrate inhibition assay Eight monosaccharide inhibitors and rabbit RBCs were used in the inhibition study (Table 2-4).

GlucNAC blocked H.A activity in Iranshahr blood-unfed females, completely. Glucose, galactose, glucNAC and fucose inhibited the midgut lectin activity of Kazerun blood-unfed females (Table 4).

Table 2: Carbohydrate inhibition of midgut extracts agglutination of erythrocytes. Degree of inhibition, compared to positive control

Kazerun population	HA	Glu.	Gal.	Lact.	Gal-NAc	Gluc-NAc	Man.	Arab.	Fuco	Cont.
Male	3 (8)*	1-2 (2-4)	3 (8)	3 (8)	1-2 (2-4)	3 (8)	3 (8)	2 (4)	2 (4)	-
Unfed female	3 (8)	0	0	3 (8)	2 (4)	0	3 (8)	1-2 (2-4)	1-2 (2-4)	-
Fed female	5-6 (32-64)	3 (8)	2-3 (4-8)	3-4 (8-16)	3-4 (8-16)	2 (4)	3-4 (8-16)	3-4 (8-16)	3-4 (8-16)	-

The numbers in parentheses show endpoint titers expressed as the reciprocals of the dilutions (1/n)

* The number of wells that hemagglutination was seen

The lowest midget extract titers were found against rabbit RBCs in Bandar-abbass 1:4 in males, 1:4in blood-unfed females and 1:16 in blood-fed females (Table 2). In Bandar-abbass population carbohydrate-specifity pattern was different between blood fed and sugar-fed females. Male midgut lectin was blocked by glucose. Blood-fed midgut lectins were heterogeneous because were inhibited by different carbohydrates (Table.2).

Table 3: Carbohydrate inhibitions of midgut extract agglutination of erythrocytes. Degree of inhibition, compared to positive control (H.A).

Bandar-Abbas Population	H.A	Glu	Gal	Lac	Gal-Nac	-Gluc Nac	Man	Ara	Fuc	Control
Male	1-2 (2-4)*	1 (2)	1-2 (2-4)	1-2 (2-4)	1-2 (2-4)	1-2 (2-4)	1-2 (2-4)	1-2 (2-4)	1-2 (2-4)	-
Unfed female	2 (4)	2 (4)	2 (4)	2 (4)	2 (4)	1-2 (2-4)	2 (4)	2 (4)	1-2 (2-4)	-
Fed female	3-4 (8-16)	0	1 (2)	2 (4)	2-3 (4-8)	2-3 (4-8)	3-4 (8-16)	4 (16)	3-4 (8-16)	-

The numbers in parentheses show endpoint titers expressed as the reciprocals of the dilutions (1/n)

* The number of wells that heamagglutination was seen

Male midgut lectin of Iranshahr population was reduced by different carbohydrates such as GlucNAC, glucose, galactose and lactose reduced hemagglutination titers endpoint one well less.

Table 4: Carbohydrate inhibitions of midgut extract agglutination of erythrocytes. Degree of inhibition, compared to positive control (H.A).

Iran-Shahr Population	HA	Glu	Gal	Lac	GalNAc	GlucNAc	Man	Arb	Fuc	Control
Male	2 (4)*	1 (2)	1 (2)	1 (2)	1 (2)	2 (4)	2 (4)	1-2 (2-4)	1-2 (2-4)	-
Unfed female	2 (4)	1-2 (2-4)	2 (4)	2 (4)	2 (4)	0	2 (4)	2 (4)	2 (4)	-
Fed female	4 (16)	2-3 (4-8)	2 (4)	2 (4)	2 (4)	2 (4)	4 (16)	1 (2)	2-3 (4-8)	-

The numbers in parentheses show endpoint titers expressed as the reciprocals of the dilutions (1/n)
* The number of wells that hemagglutination was seen

Discussion

The midgut lectin activities and egg-float ridge number were investigated in three geographical population of *An stephensi* (Bandar-abbas, Kazerun and Iranshahr). All the specimens were collected from rural areas and colonized in insectaries.

Bandar-Abbas population was categorized as the Intermediate group (Egg-float ridges number= 14, 15, 16), Iranshahr in the Intermediate group with a tendency to Mysoransis (EFRN= 11, 12, 13, 14, 15) and Kazerun in the Intermediate group with a tendency to Type form (EFRN = 13, 14, 15, 16, 17).

Type form variant is a strong vector in urban areas and Mysoransis variant is a poor vector in rural localities (22, 23). Chen & Billingsley (20) characterized two lectins from heamolymph of *An stephensi* in. A mannan-binding protein that agglutinated human & sheep RBCs was detected from female heamolymph. A galactose, lactose and heparin specific lectin was detected in male mosquitoes that could agglutinate rat, horse, rabbit and calf RBCs. (22). Lectins and lectinoids are ubiquitous molecules and exist in plants, animals and microorganisms. Insects have specific lectins against various RBCs in their heamolymph and other tissues (7).

In present study, we investigated the presence of midgut hemagglutinins in malaria vector *An. stephensi* from south and southeast of Iran. Midgut lectin in this species agglutinated rabbit RBCs but not human, rat and sheep. No lectin activity was found in larvae and pupa.

Variations in agglutination activity in both male and female mosquitoes, within and between populations were detected. The endpoint titers were very similar in male and blood-fed females but carbohydrate-binding pattern was different which suggests the sexual dimorphism in midgut lectin type in this species.

The other important issue is secreted lectins after a blood meal. Carbohydrate binding pattern of midgut lectins is different between blood-fed and blood unfed females in all three populations and this may be due to secreted lectins after a blood meal (blood feeding induced lectins).

Kazerun population of *An. stephensi* had the highest hemagglutination titers among all three populations, and also the highest egg-float ridges number belonged to this population, this outcome suggests that maybe in strong vectors, the quantity and concentration of midgut lectin is might be higher.

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