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Standardization of enzyme linked immunosorbent assay for the detection of antibodies to *Argas persicus* (Oken, 1818) infestation in fowl of Telangana State

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Abstract
In the present study the checker board titration was applied for optimization of Goat anti-chicken IgY HRPO conjugate, *Argas persicus* salivary gland antigen concentration and serum dilution. In the present study, the optimum conjugate dilution was 1:3000, optimum antigen concentration was 1 µg and optimum serum dilution was 1:100. The overall sensitivity of ELISA was 84.78% (39 out of 46 true positives) and the specificity as 70% (7 out of 10 true negatives). The laboratory standardized ELISA was applied on chicken sera collected from different poultry farms located in and around Hyderabad. A total of 486 chicken sera (326 layers and 160 backyard chicken) were tested out of which 125 (119 commercial layers plus 6 backyard chicken) were found positive indicating 25.72% (36.50% in commercial layers and 3.75% in backyard chicken) of prevalence respectively. A significant difference existed between the incidences among layers and backyard chicken.

Keywords: *Argas persicus*, Antibodies, ELISA, Chicken

1. Introduction
The fowl tick, *Argas persicus* was first described in 1818 by Lorenz Oken in Meyaneh, Persia and are now established as having a worldwide distribution, particularly affecting warmer regions because of its close association with *Gallus domesticus*. *Argas persicus* (Oken, 1818) has originated in Central Asia [1] and a specific pest of domestic and certain wild birds in parts of Asia [2, 3], Africa [4], Australia [5], Europe and America [6]. The fowl tick is hiding in the cracks, crevices of poultry house, bird nests, roosting sites and also under the bark of many trees. *Argas persicus* causes weakness and anaemia due to loss of blood, reduces egg yield, growth and cause death of birds [7], moreover, it causes paralysis in heavily infested chickens [8, 9]. *Argas persicus* is the main vector of *Borrelia anserina* [10-13] and *Aegyptianella pullorum* [11]. Very limited number of serological tests have been developed for the detection of antibodies to *Argas persicus* infestation till to date. Specific anti *Argas persicus* tick circulating antibodies were earlier detected by ELISA [14, 15]. In the present study an ELISA was used to detect the antibodies to *Argas persicus* infestation in chickens and the sensitivity and specificity of this assay were determined.

Materials and Methods
Adult male and female ticks were found in the cracks and crevices of poultry houses. Then the ticks were dissected and separated the salivary glands with fine tipped forceps and rinsed thrice in PBS (pH 7.4) and transferred in to fresh PBS (pH 7.4).

Salivary gland antigen preparation
One hundred salivary glands were homogenized in 3 ml of PBS (pH 7.4) in glass homogenizer containing 1mM PMSF. Then the homogenate was sonicated at 20 Watts of 6 cycles each lasting for 30 seconds with an interval of 30 seconds in between. The suspension thus obtained was centrifuged at 18,000 × g in a refrigeratory centrifuge at 4^0C for 30 minutes. Then the supernatant was collected and protein concentration was determined. The salivary gland antigen was made in to aliquots and stored at -20 ^{0}C until used.

Reference Sera
The serum samples collected from chickens found positive for *Argas persicus* infestation on gross examination and with no other ectoparasites in different poultry farms were treated as known positive sera. Similarly, the sera collected from day old chicks found negative to *Argas*

persicus infestation were treated as known negatives in all test procedures. Merthiolate was added at a ratio of 1:10,000 to the serum samples as a serum preservative and stored at -20 ^{0}C until further use.

Enzyme Linked Immunosorbent Assay
A checker board titration was performed to determine the working strength of Goat Anti Chicken IgY HRPO conjugate, antigen concentration and serum dilution [16].

Determination of working dilution of Goat Anti chicken IgY HRPO conjugate
To determine the working dilution of Goat anti chicken IgY-HRPO conjugate, 50 µl of normal chicken sera (1:100) in 0.2 M Carbonate-Bicarbonate buffer (pH 9.6) was coated on to 96 well flat bottomed ELISA plate and incubated at 37 ^{0}C for 1 hour. The ELISA plate was washed thrice with washing buffer each for 5 min. The blocking buffer was added to block the non-specific reactive sites and incubated at 37 ^{0}C for 1 hour followed by washing as described earlier. Test conjugate dilutions (1:1000, 1:2000, 1:3000, 1:4000 and 1:5000) were prepared in washing buffer and 50µl of each dilution was added to the wells in duplicate and incubated at 37 ^{0}C for 1 hour. The plate was washed again as before. Fifty microliters of Ortho Phenylene Diamine substrate (OPD) was added to each well and incubated in darkness for 15 min. The reaction was stopped by adding 2.5 M H_2SO_4 and the absorbance was recorded by using Multiskan Labsystems ELISA Plate Reader at 492 nm.

Determination of optimal concentration of antigen
To determine the optimal concentration of antigen, 50 µl of different dilutions of antigen (4µg, 2µg, 1µg, 0.5µg, 0.25µg, 0.125µg, 0.0625µg) in 0.2 M Carbonate-Bicarbonate buffer (pH 9.6) was added to 96 well flat bottom ELISA plate. One row was used for each dilution of antigen and the plates were incubated at 37 ^{0}C for 1 hour for coating of antigen. The plate was washed with washing buffer thrice each for 5 min. Then 50 µl of blocking buffer (5% skim milk in PBS, pH 7.4) was added to each well to block the unbound sites and incubated at 37 ^{0}C for 1 hour. Then the plate was washed with washing buffer thrice each for 5 min. Different dilutions of both positive and negative chicken sera starting from 1:50, 1:100, 1:200, 1:400 and 1:800 were prepared in PBST and 50 µl of each dilution was added to each well (column wise) and incubated at 37 ^{0}C for 1 hour followed by washing with washing buffer thrice each for 5 min. Remaining procedure was described in the conjugate dilution.

Determination of optimum serum dilution
To determine the optimum serum dilution, 50 µl of previously determined optimum concentration of antigen (1µg) in 0.2 M carbonate bicarbonate buffer (pH 9.6) was added to each well of 96 well flat bottom ELISA plate. The plates were incubated at 37 ^{0}C for 1 hour for coating of antigen. Then the plate was washed with washing buffer thrice each for 5 min. The blocking buffer (5% skim milk in PBS) was added to block the non-specific reactive sites and incubated at 37 ^{0}C for 1 hour. Then the plate was washed with washing buffer as described earlier. Different dilutions of both positive and negative chicken sera starting from 1:50, 1:100, 1:200, 1:400 and 1:800 were prepared in PBST and 50 µl of each dilution was added to each well (column wise) and incubated at 37 ^{0}C for 1 hour and then washed with washing buffer as described earlier. Remaining procedure was described in the earlier.

Determination of cut off value
10 serum samples negative for *Argas persicus* infestation at optimum serum dilution (1:100) were tested using 1 µg of antigen per well and their mean and standard deviation were calculated. As the performance of serological assay is reported to be improved by adding two times the standard deviation to the mean OD values of the negative controls [17], the cut-off value was calculated by using Mean+2SD for deciding the status of sera samples with respect to salivary gland antigen. Samples with OD value above this cut-off point was considered as positive.

Sensitivity and specificity of ELISA
The sensitivity of ELISA was measured by screening 46 sera samples collected from *Argas persicus* infected birds. The specificity of laboratory standardized ELISA was measured by screening 10 sera samples of day old chicks which were found negative for the *Argas persicus* infestation on gross examination.
The sensitivity thus arrived was measured by the formula

$$\text{Sensitivity: } \frac{\text{True positive} \quad X \quad 100}{\text{True Positive + False Negative}}$$

The specificity thus arrived was measured by the formula

$$\text{Specificity: } \frac{\text{True negatives} \quad X \quad 100}{\text{True negatives + False Positives}}$$

Statistical Analysis
Statistical analysis was performed by test of proportions as described by Snedecor and Cochran (1989). Chi-square test of association was used. *P* value less than 0.01 was considered as significant.

Results
Extraction of antigen
The protein concentration in salivary gland antigen was estimated by Nano spectrophotometer. The protein concentration of salivary gland antigen obtained was 288 µg/ml.

Enzyme Linked Immunosorbent Assay
Optimization of Goat Anti-chicken IgY HRPO conjugate
Conjugate at 1:1000 dilution gave an optical density (OD) value of 2.132, while the end dilution of 1:5000 conjugate gave an OD value of 1.103. There was a sudden drop in OD values (Fig. 1) for conjugate dilutions above 1:3000. Therefore, the dilution 1:3000 of the conjugate was used as optimum working dilution in all ELISA procedures in the present study.

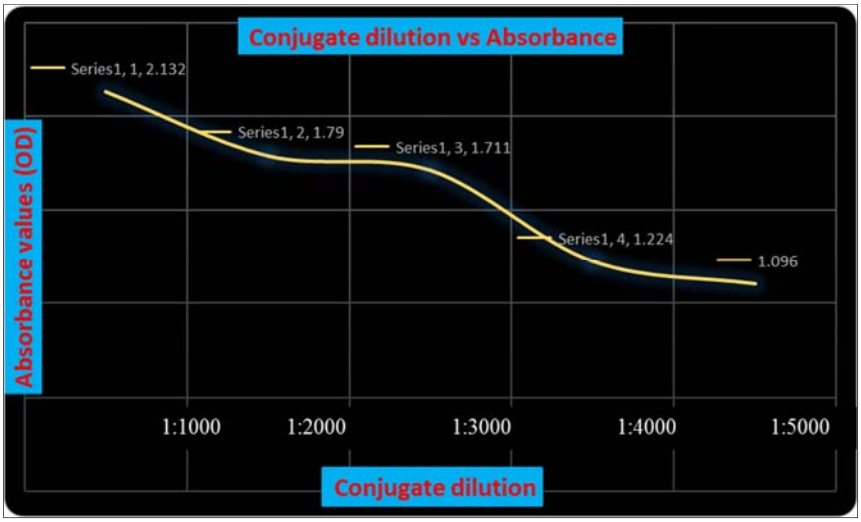

Fig 1: Graph showing the optimization of Goat Anti-chicken IgY HRPO conjugate with serum dilution (1:100).

Optimization of *Argas persicus* salivary gland antigen concentration

Checkerboard titrations were carried out to find the optimum concentration of *Argas persicus* salivary gland antigen against both positive and negative serum. The OD value obtained at 1.0 µg was 1.280. There was a sudden drop in OD values for antigen concentration above 1.0 µg (Fig. 3, 4). Therefore, the concentration of 1.0 µg of the antigen was used as optimum working dilution in all ELISA procedures in the present study.

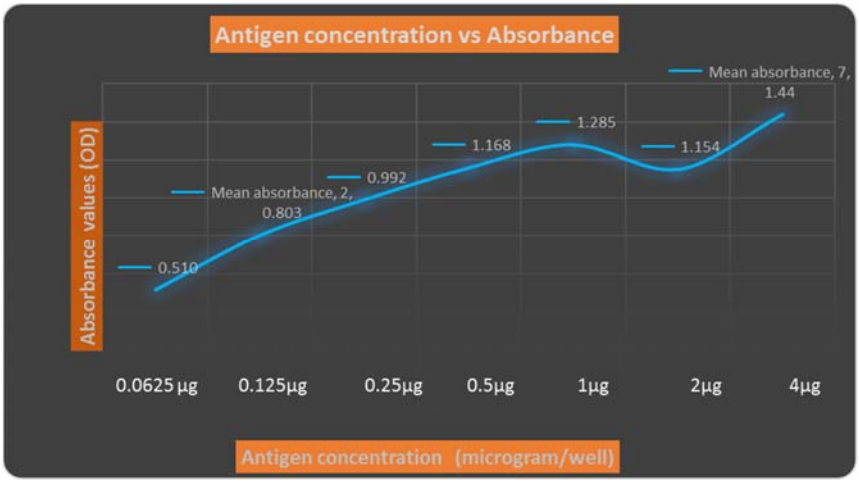

Fig 2: Graph showing the optimum antigen concentration against positive serum.

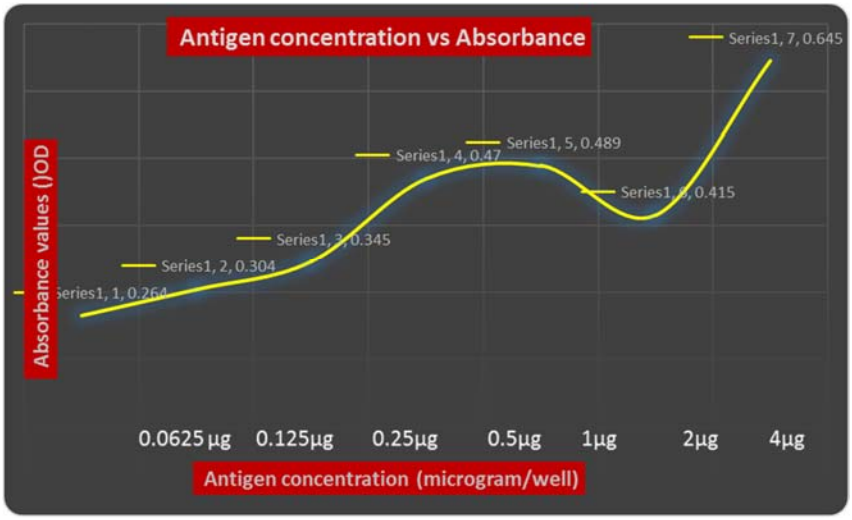

Fig 3: Graph showing the optimum antigen concentration against negative serum.

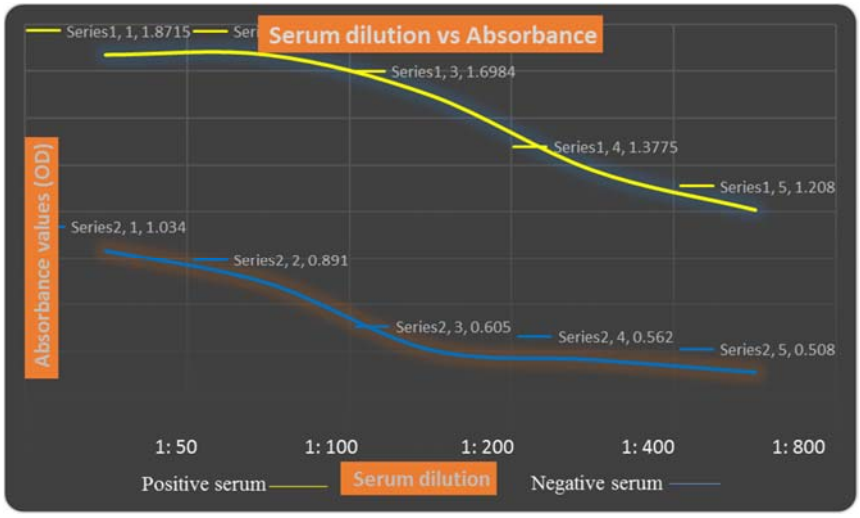

Fig 4: Graph showing the reactivity of positive and negative sera with 1 µg antigen.

Optimization of serum dilution

The mean OD value at 1:100 dilution of chicken *Argas persicus* positive serum was 1.8710. There was a steep fall in OD values of positive sera above 1:100 dilution. Therefore, the 1:100 dilution was considered for fixing optimum dilution of positive sera in the present study (Fig. 3). As there was a steep fall in the OD values of negative sera above 1:100 dilution (Fig. 3, 4).

Determination of cut off value

For this purpose, 10 negative sera samples at optimum dilution (1:100) were tested. They had a mean OD of 0.770 with a standard deviation (SD) of 0.139. The cut-off value was adjusted to 1.048 (Mean +2SD) and the samples which gave OD above this value were considered as positive for *Argas persicus* antibody.

Sensitivity and specificity of ELISA

The overall sensitivity of ELISA was determined as 84.78% (39 out of 46 true positive) and shown in Table. 1 and specificity was 70% (7 out of 10 negative sera) and depicted in Table. 2.

Table 1: Sensitivity of ELISA in detecting antibodies to the salivary gland antigen of *Argas persicus* in chicken

Sl. No.	Antigen	Number of samples (Known positive)	ELISA (Positive)	ELISA negative (False negative)	Sensitivity Percentage
1	Salivary gland antigen	46	39	7	84.78

Sensitivity: True positive/True Positive +False Negative X100

Table 2: Specificity of ELISA in detecting antibodies to the salivary gland antigen of *Argas persicus* in chicken

Sl. No.	Antigen	Number of samples (Known negative)	ELISA (Negative)	ELISA positive (False positive)	Specificity percentage
1	Salivary gland antigen	10	7	3	70

Specificity: True negatives/True negatives + False positives X100

Efficacy of standardized ELISA for detection of antibodies against *Argas persicus* infestation in field cases

The results of ELISA to detect specific antibodies against *Argas persicus* salivary gland antigen is presented in Table. 4.7. A total of 486 chicken sera (326 layers and 160 backyard chicken) were examined by ELISA out of which 119 layers and 6 backyard chicken were found positive indicating 36.50% and 3.75% of layers and backyard chicken respectively were infested with the tick species.

Statistical Analysis

Chi-square test indicated significant infestation of *Argas persicus* of layers and backyard chicken at *p*- value < 0.00001.

Discussion

In the present study the protein concentration was 288 µg/ml. A protein concentration of 43 mg/ml from salivary gland extract of *Boophilus annulatus* [18]. The low protein (288 µg/ml) concentration obtained in our study compared to other workers could be due to less number of salivary glands (100/3 ml) used in the extraction of protein. The wide variation in protein concentration achieved by the above researcher might be due to different techniques used in the purification of protein and different methodologies used in protein estimation.

The Goat anti-chicken IgY HRPO conjugate was optimized at a concentration of 1:3000 against 1:100 diluted healthy chicken serum [16] which was higher than the conjugate dilution (1:2000) [14] who used larval antigen to detect anti-tick circulating antibodies in serum. This could be due to the kind of antigen (larval antigen) and type of conjugate (anti-chicken IgG (whole molecule) peroxidase conjugate) used in his study.

The antigen concentration was standardized at 1.0 µg per reaction by using checker board titration which was similar to the findings of [14] and [19]. The optimum antigen concentration arrived in our study was lower [20-24]. The wide variation in optimization of antigen concentration achieved by above researchers could be due to the presence of non-specific proteins.

Efforts were also made to achieve the end results within shortest possible time period by reducing the antigen incubation period to 1 hour at 37 ^{0}C. This observation was an improvement over the protocol followed by [20, 21, 24] who incubated the same for overnight at 4 ^{0}C.

We could achieved a positive result at a highest serum dilution of 1:100. The OD value at 1:100 dilution of anti *Argas persicus* positive and negative sera obtained against 1.0 µg of *Argas persicus* salivary gland antigen concentration was 1.8710 and 0.8910, respectively. The optimum sera dilution reached in our study was similar to the findings [19, 22] but not coinciding with the observations [20, 21, 24]. The sensitivity and specificity findings could not be compared due to paucity of literature on immunodiagnostic studies on *Argas persicus* by ELISA.

The comparatively higher incidence recorded in commercial layer chicken could be due to confined rearing of birds in poultry house where recycling of infestation is possible. Whereas a very low incidence of tick infestation in backyard chicken might be due to possibly lesser recycling of tick infestation and also due to naturally acquired immunity compared to commercial layer chicken.

In the present study the prevalence rate in backyard chicken and commercial layers were higher than the findings [7, 25]. This might due to the climatic conditions such as relative humidity, temperature and other factors which favour the tick growth and multiplication. The highest prevalence in poultry farms [26, 27] whereas higher prevalence in backyard chicken [28] were reported when compared to our findings. This might be due to the high relative humidity and optimum temperature for growth of ticks at different places of study.

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