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Dynamics of antibody titres in dogs vaccinated with a polyvalent killed *Leptospira* vaccine as measure for quality control

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Leptospirosis, an important disease of dogs of all breeds, is characterized by vomiting, dehydration, blood stained faeces, nephritis, shivring, jaundice and death (Verma and Srivatava *et al.* 1998). A number of firms are importing canine vaccines in India, but there is a lack of quality control of these vaccines. This paper reports quality control of an imported vaccines used to vaccinate dogs based on measurement of antibody titres using microscopic agglutination test (MAT) and latex agglutination test (LAT).

Bacterial cultures and media: Eleven different serovars of *Leptospira* comprising *L. interrogans* serovars Canicola, Pomona, Icterohaemorrhagiae, Australis, Autumnalis, Pyrogenes, Patoc, Javanica, Hebdomadis, *L. kirschneri* serovar Grippotyphosa and *L. borgpetersenii* serovar Hardjoprajitno were employed for conducting MAT. These leptospiral serovars are being maintained in Ellinghausen McCullough Johnson (EMJH) Harris media at *Leptospira* Laboratory, Division of Bacteriology and Mycology, IVRI, Izatnagar (Uttar Pradesh).

Serum samples: Puppies (102) belonging to German shepherd breed administered with a killed vaccine that protects against 4 most common serovars *Leptospira interrogans* serovars Grippotyphosa, Pomona, Icterohaemorrhagia. This vaccine incorporated states of subunit technology. The antibody titer was detected by MAT and by rLipL32LAT during the 16 weeks of post vaccination period.

Microscopic agglutination test (MAT): The serum samples collected from vaccinated dogs were tested by MAT (Faine 1982) for detecting antibodies using 11 different leptospiral serovars mentioned above. Sera were diluted to 1:50 using PBS and 40 µl of each of the diluted serum was transferred to 96 well microtitre plate and an equal volume of live 5-day-old *Leptospira* culture containing approximately 2.4 × 10^8 cells/ml was added. The plates were examined by dark field microscopy after 3–4 h of incubation at 37°C for agglutination of *Leptospira* cells.

Preparation of recombinant protein antigens LipL32: The freeze-dried ampoules of recombinant *E. coli* carrying clone of LipL32 antigen of *L. interrogans* serovar Canicola available in Leptospira Laboratory, Division of Bacteriology and Mycology, IVRI, Izatnagar were suspended into 5 ml of liquid L.B media containing 5 microlitre ampicillin (1µl/1ml) and incubated at 37°C overnight. The growth of the culture was checked by observing the density of the culture. This process was repeated 2–3 times for obtaining pure growth of the culture. Subsequently, this clone was grown in 5 ml LB-ampicillin (100µg / litre) broth overnight at 37°C under constant shaking and 100 µl of it was inoculated into fresh 10 ml LB medium. The culture was grown for 10–12 h to an OD-600 of 0.6–1 and then 1 mM IPTG was added to it and further incubated under shaking at 37°C for overnight. Next day the aliquots of 1ml from IPTG induced culture were collected and the pellets obtained after centrifugation at 8000 rpm for 10 min were re-suspended in 50 µl of 2X Laemmli buffer and analyzed using SDS-PAGE. For large production of recombinant clones, the cells were grown in 1 litre of LB broth with ampicillin and the expressed protein was confirmed by SDS-PAGE. The polyhistidine (6X-His) tagged fusion protein was purified under denaturing condition by nickel chelating affinity chromatography. This recombinant protein was dialyzed and the concentration was determined by Lowry's *et al.* (1951).

Latex agglutination test (LAT): Latex agglutination test was performed according to Srivastava *et al.* (1989). The commercially available latex particles were coated with recombinant *Leptospira* antigens. Briefly, the recombinant LipL32 protein was dialyzed against glycine buffer (pH 8.3) overnight at 4°C. The latex beads (0.8µm diameter, 2.5% solution) were washed thrice with glycine buffer (by centrifuging at 6000 rpm for 30 min each time) and finally

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suspended in the same buffer. The washed 400 µl latex beads were mixed with 1 ml of dialyzed recombinant LipL32 protein (500µg/ml) and incubated at 37°C in shaking water bath for 3–5 h to coat the antigen. Subsequently, the antigen coated beads were stored at 4°C overnight. To standardize the test, one drop (5–10µl) of antigen coated latex beads was mixed with a drop of known positive anti *Leptospira* antibodies serum sample on a clean glass slide and observed for agglutination reaction, if any. If the reaction was not good, the antigen coated beads were washed with 0.1% BSA (in glycine buffer) and again the test was repeated with known positive and known negative serum samples. Once the tests, viz. rLipL32-LAT and rLipL41-LAT got standardized, all the field sera were screened for presence of anti *Leptospira* antibodies employing these tests.

Evaluation of LAT and MAT: The relative sensitivity, specificity and accuracy of the rLipL32 and rLipL41 based LAT for serodiagnosis of leptospirosis were evaluated in comparison to MAT as described below:

$$\text{Relative sensitivity} = a/(a+c)\times 100$$

where, a is the number of sera positive both by LAT and MAT, while c is the number of sera positive by MAT but negative by LAT.

$$\text{Relative specificity} = d/(b+d)\times 100$$

where, d is the number of sera negative both by LAT and MAT, while b is the number of sera negative by MAT but positive by LAT.

$$\text{Accuracy} = a+d/a+b+c+d \times 100.$$

All the dog serum samples collected were subjected to MAT, rLipL32-LAT for the presence of *Leptospira* antibodies. Of the 102 dog serum samples, MAT detected antibodies in 71 samples (69.60%) against 4 serovars, viz. Pomona, Icterohaemorrhagiae, Canicola, Grippotyphosa, Javanica, Australis and Autumnalis. Majority of sera (65) showed varying degrees of titre to serovar Pomona followed by Icterohaemorrhagiae (34), Canicola (32), Grripotyphosa (11) and Javanica (9). Recombinant LipL32LAT 92 (90.19%) samples as positive at 16 weeks, out of 32 sera 22 were found positive for MAT and 30 were found positive for rLipL32LAT. At 16 weeks post vaccinated titer 10 sera showed highest titer 1:60. Out of 10 sera 2 showed highest titer for serovar Icterohaemorrhagiae and Pomona, 6 showed for Pomona, 1 for Canicola, Pomona and Grippotyphosa, 1 for Pomona and Grippotyphosa. At 12 weeks, out of 17 sera 10 were found positive for MAT and 15 were found positive for rLipL32LAT. At 12 weeks sera only one sera showed highest titer (1:60) for Pomona. At 5 weeks, out of 38 sera 27 were found positive for MAT and 32 were found positive for rLipL32LAT. At 5 weeks 11 sera showed highest titer (1:40) only for Pomona and 1 sera showed highest titer for all the four serovar Icterohaemorrhagiae, Canicola, Pomona and Grippotyphosa. At 4 weeks, out of 15 sera 11 were found positive for MAT and all 15 were found positive for rLipL32 LAT. At 4 weeks sera only 2 sera showed highest titer for serovar Pomona.

There is a very little contemporary literature available on the aspect of quality control of *Leptospira* vaccines for canines. Barr *et al.* (2005) studied serological responses of dogs given a commercial subunit vaccine against *Leptospira interrogans* serovar Pomona and *Leptospira kirschneri* serovar Grippotyphosa and concluded that- Subunit vaccines against serovar Pomona and Grippotyphosa induce MAT titers not only in homologous antigens but also to serovar Autumnalis, which could lead to a misdiagnosis of leptospirosis caused by serovar Autumnalis. This study warrants that immune response to vaccination of dogs with indigenous or imported vaccines be monitored as part of quality control of vaccines. In leptospirosis, predominant serovars are incorporated into the vaccine but the constraint remains that new serovar be added into the vaccine in case of its emergence of any new as there is no cross protection between serovars. As far as the antibody titre is concerned, it was seen in this study that a titre of 1:60 can not be considered a high titre. In vaccinated animals the titre should be sufficiently high. The USDA recently developed and validated *in vitro* enzyme-linked immunosorbent assay (ELISA) antigen quantification methods for potency determination of vaccines for several *Leptospira* serogroups (i.e., *Leptospira interrogans serogroups pomona, canicola, icterohaemorrhagiae*, and *Leptospira kirschneri serogroup grippotyphosa*) (*iccvam.niehs.nih.gov/.../LeptoWkspAgenda-Prog-Final-508.pdf, 2012)*. In India, there is a need to prioritize the research, development and validation of *Leptospira* vaccines in dogs.

SUMMARY

Dynamics of antibody production in post vaccination sera of 102 puppies belonging to German shepherd vaccinated with a commercial polyvalent *Leptospira* vaccine was monitored employing microscopic agglutination test (MAT) and latex agglutination test (LAT), using a genus specific recombinant protein antigens of Leptospira, viz. serovars Canicola, Pomona, Grippotyphosa and Icterohaemorrhagiae. Results indicated that out of the 4 serovar used in the vaccine, Pomona was highly immunogenic followed by Icterohaemorrhagiae, Canicola and Grippotyphosa in the decreasing order. Since only 69.60% of the animals showed the post immunization titres against either of the 4 serovars employed in vaccination, this warrants the quality control of vaccines.

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