The anti-peptide relaxin antibodies for monitoring the well-being of the fetus in pregnant bitches

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Pregnancy management is difficult in canines and there is a lack of methodology which would allow monitoring embryonic well-being. Relaxin (Rlx) is being reported for use in pregnancy diagnosis in different species. Here, we evaluated seven anti-Rlx peptide antibodies for detection of well-being of the fetus in bitches. Peptides were synthesized using solid phase peptide synthesis chemistry and the hyper-immune sera raised in chicken against peptides. In sandwich enzyme linked immunosorbent assay (ELISA), the chicken anti-Rlx P4 as a capture and rabbit anti-prorelaxin as detection antibody, gave better results in terms of differentiating the pregnant from non-pregnant bitches. Thirty five canine serum samples (21 non-pregnant, 11 pregnant and 3 males) were screened. Among the 11 pregnant, six delivered normally and the rest of the bitches aborted a few days after the serum collection with one or two dead fetuses. Among the 11 pregnant serum samples, 4 showed the absorbance above the cut-off value set for pregnancy, which delivered healthy puppies and five bitches showed the absorbance below the set cut-off value absorbed after a few days of blood collection with one or two dead fetuses. The specificity of the assay was found to be 90.47% and the sensitivity of the assay for detecting the well-being of the fetus is 82%. The serum samples collected from those bitches having problems related to normal whelping, had shown absorbance below the set cut-off value. The results of the study indicate that relaxin is a useful marker, specifically for monitoring the pregnancy and tells about the well-being of the fetus.

Keywords: Canine, relaxin peptide, sandwich ELISA, pregnant bitch, fetus, well-being

Introduction

The well-being of the fetus is important for management of pregnancy in any species including dogs. Till date in the bitch, physical examination, ultrasound and vaginal exfoliative cytology remain the most common and effective method of pregnancy diagnosis. All normal bitches go through a stage of their reproductive cycle after estrus called luteal phase, or “diestrus” where the hormone progesterone is produced at elevated levels, irrespective of the animal is pregnant or not. Dogs may also be unique in their sensitivity to progesterone, which can rise to a maximum during hypertrophy and hyperplasia of the uterus. There will be a pre-ovulatory rise in serum progesterone associated with the pre-ovulatory lutenization of follicles. These facts make progesterone unreliable for pregnancy diagnosis in bitches². Other reproductive hormones like prolactin and luteinizing hormone (LH) are not considered as suitable marker for pregnancy diagnosis because of diurnal variations¹. There are reports of elevated levels of acute phase proteins like C-reactive protein, serum amyloid A, haptoglobin, alpha-1-acid glycoprotein and ceruloplasmin in pregnancy. But elevated levels of acute phase protein are also seen in most instances of inflammatory diseases, tumor cases and those in pregnancy may represent a similar response to implantation³. There are reports of peptide hormone called relaxin (Rlx) to be exclusively produced in pregnant bitches and not by non-pregnant and pseudo-pregnant bitches⁴⁵. The fetal heart rate by ultrasonography is routinely practiced by clinicians for assessing the well-being of the fetus. However, there are no reports of any marker protein for well-being of the fetus.

The relaxin (Rlx) is a 6 kDa peptide hormone belongs to insulin like growth factor family. The chemical synthesis or expression of the hormone in an active form is a tough task as it is synthesized as a single-chain precursor, pre-prorelaxin that is
processed by cleavage of the signal peptide and by internal cleavage of a connecting peptide to form two disulfide-linked chains to form the active hormone. The major sources of circulating Rlx varied among species\(^6\)-\(^{11}\). In dogs, both placenta and ovary secrete Rlx, with the placenta being the major contributor to the serum Rlx levels during pregnancy\(^5\). Relaxin has a variety of actions associated with reproduction, such as remodeling of the reproductive tract during pregnancy\(^{12\text{-}13}\), growth of the interpubic ligament uterine growth\(^{14}\) and softens the uterine cervix before labor\(^{15}\). Relaxin also stimulates human sperm motility\(^{13}\), inhibition of myometrial contractility thereby preventing preterm birth of a baby\(^{16}\). It is also implicated in the preparation of the endometrium for fetal nourishment and in uterine accommodation of the developing fetus. Remodeling of uterine stroma and vasculature and production of factors that promote trophoblast adherence to and invasion of endometrial\(^{17\text{-}20}\). Circulating concentrations of placental Rlx are elevated from approximately 21–24 d after the LH surge to the end of pregnancy\(^{21}\). Serum Rlx concentrations are not detectable at any time in non-pregnant (diestrus) bitches. In pregnant bitches, it reaches peak concentrations (5 ng/ml)\(^1\) to 50 ng/ml\(^5\) in late pregnancy (40–50 d of gestation). Rlx concentrations decrease after parturition, but remain detectable for at least 30 days during lactation\(^{1,14}\). The source of relaxin during lactation remains unclear. However, it is speculated to cause the mammary gland growth\(^{22\text{-}23}\).

Canine Rlx is of particular interest for being a pet animal and has been extensively studied. A radioimmunoassay using a biologically active synthetic peptide has been developed for canines\(^24\) which has formed the basis of a commercially available Rlx assay (ReproCHEKTM) for diagnosis of pregnancy in bitches. The chemical synthesis of the Rlx peptide in its active form in a sufficient amount and affordable cost has still remained as a challenge. Though, the Rlx assays are employed for pregnancy diagnosis, the results of early pregnancy are inaccurate or needed to confirm by second sampling. Our preliminary studies in a bitch, though pregnant, the Rlx level had been at basal level and at a later stage the bitch got aborted. Hence, the present study was undertaken to evaluate Rlx peptide as a biomarker for the well-being of the fetus in canines rather as pregnancy specific protein.

### Materials and Methods

#### Biological Materials

The blood samples were collected from the cephalic vein of male and dry female non-pregnant dogs housed separately in Experimental Shed of the Animal Nutrition Division of Indian Veterinary Research Institute, Bareilly (U.P.). Few blood samples were also collected from Referral Veterinary Polyclinic of the Institute and different breeders in Bareilly (U.P.), India. The usage of the materials and method are duly approved by the Institute Animal Ethics committee. Serum was separated and preserved at –20°C until use.

#### Solvents for Peptide Synthesis

The dried dimethylformamide (DMF), dichloromethane (DCM), dried ethanol, methanol and diethyl ether were of analytical grade and obtained from SD-Fine Chemicals (India). The dimethyl amino pyridine (DMAP), piperidine and thioanisol were from Spectrochem (India). trifluoroacetic acid (TFA) and N, N-diisopropoylcarbodiimide (DIPC) were used from Sigma (USA). The coupling reagents such as 1-hydroxy benztriazole (HOBT) was from Orpegen Pharma (Germany), 2-(1H-benzotriazolyl-1-yl)-1,1,3,3-tetramethlyuronium hexafluoro phosphate (HBTU) was purchased from GL Biochem (Shanghai, China) and Nova Biochem (Switzerland).

#### Reagents Used for Conjugation

The KLH used for conjugation procured from Sigma, and N-hydroxysuccinimide (NHS) and N-ethylcarbodiimide (EDC) were from Sigma (USA).

#### Prediction of Epitope

Five different epitopes of both chains A and chain B have been identified using IEDB analysis resources ([http://tools.immuneepitope.org/bcell/](http://tools.immuneepitope.org/bcell/)) using the sequences of Rlx A and B chains as reported by Stewart and co-workers\(^25\).

#### Solid Phase Peptide Synthesis

Peptide synthesis using standard 9-fluorenylmethoxy-carbonyl chemistry devised by Merrifield (1963) had been used for Rlx peptide synthesis\(^{26}\). The successive addition of Fmoc amino acids was carried by using HOBT-HBTU on Wang resin with 0.9 mmol/g loading efficiency of 100-200 µm size of beads.

#### (i) Loading of First Fmoc-Amino Acid to Wang Resin

The first Fmoc-amino acid protected at the side chain was coupled to Wang resin. The 100 mg...
of Wang resin beads was taken in modified Merrifield apparatus and sufficient amount of dimethylformamide (DMF) was added and allowed to swell for overnight. Relative to the loading efficiency of resin, first Fmoc-amino acid, i.e., from the C-terminal end was dissolved separately in 5 ml of dry dimethylformamide and cooled over icebath. Same amino acid was activated with N,N-diisopropylcarbodiimid (DIPC) (3 equivalent) and dimethyl amino pyridine (DMAP) (0.1 equivalent) in dimethylformamide and was kept at 0°C for 15 min. Then this solution was added to Wang resin in the reaction vessel and stirred for 2 h at room temperature in a shaker at 160 rpm. Subsequently, Wang resin was washed 5 times with DMF and 5 times with dichloromethane (DCM) to remove excess amino acids.

(ii) Estimation of Loading Efficiency of First Amino Acid
From the reaction vessel, 20 mg of Wang resin was taken and washed 5 times with dry diethyl ether and dried under vacuum. About, 1 mg of dried Wang resin was added to a 3 ml of 20% piperidine solution and kept for 5 min with occasional swirling. The resin was allowed to settle at the bottom. The supernatant was taken inside silica UV cell of 10 mm path length and the absorbance at 290 nm was measured by comparing against 20% piperidine solution. The average value of Fmoc amino acid loading into resin obtained using standard reference.

(iii) End Capping of Unreacted Ends of Resin
The unreacted functional groups on Wang resin were blocked to avoid formation of truncated peptide fragments. About 500 µl of end capping solution (DMF : acetic anhydride : DIEA (193:6:1) was added to above vessel and stirred in shaker for 15 min at 150 rpm. The beads were washed 3 times each with DMF and once with DCM and again twice with DMF. The Wang resin with first amino acid was ready for next amino acid coupling.

(iv) Deprotection of Fmoc from N-Terminal
For addition of subsequent amino acid, deprotection of amino group was done. The Fmoc amino acid-Wang resin was treated twice with 20% piperidine in DMF (v/v) and stirred in shaker at 25°C for 15 min at 150 rpm. The beads were washed 3 times with DMF.

(v) Successive Coupling of Next Amino Acids
The Wang resin was taken in dry DMF. Fmoc-amino acid (2nd amino acid) was taken at the rate of 3 equivalent and dissolved in dry DMF, subsequently, this amino acid was activated with 3 equivalent HOBt and 2.9 equivalent HBTU and kept at 0°C for 15 min. Followed by the addition of Wang resin in the reaction vessel and stirred for 2 h in a shaker at room temperature at 160 rpm. Unreacted amino acids and other reagents were washed off twice with DMF. The coupling was checked using Kaiser test. In case of negative reaction, coupling procedure was repeated without removing an Fmoc group from the added amino acid on Wang resin. After successful coupling and deprotection, next amino acid was added by repeating all the steps of deprotection, coupling and end-capping to get peptide of desired length. Finally, excessive reagent was removed and resin was washed thoroughly with DMF and then dried by washing with dry methanol and stored in desiccators in dried form until further use.

(vi) Final Deprotection and Cleavage of Peptide from Resin
Dried resin bound to peptide was taken into an Eppendorf tube. Cleavage solution containing TFA : water : thioanisole : phenol : EDT (92 : 2 : 2 : 2 : 2) was added @ 200 µl/ tube to submerge the peptide bound resin and kept on vortex for 4 h. Subsequently, the tubes were centrifuged at 10,000 rpm at room temperature for 5 min and TFA extract containing cleaved peptide was collected and precipitated by pouring dry, chilled diethyl ether in separate tubes and washed five times with diethyl ether and subjected to vacuum drying. The white powder thus obtained was stored under dry condition in desiccator until further use.

Multiple Antigenic Peptide (MAP) Synthesis
Peptide sequence was synthesized in 2 armed MAP format with cysteine at the C-terminal and lysine mosaic.

Coupling of Fmoc-Cysteine to the Wang Resin
About 100 mg of Wang resin (loading efficiency of 0.6 to 1.0 mmol/g of resin) was placed in a clean, dry frittled column with sufficient amount of DMF and allowed the resin for overnight swelling. About 175.7 mg of Fmoc-cysteine (3 equivalent to the loading efficiency of the resin) was dissolved in the DMF in the separate flask at 0°C (in ice). A solution of DIPC (37.8 mg, 3 equivalents to the loading efficiency of the resin) was added to Fmoc-cys solution and the mixture was stirred for 20 min at 0°C. Symmetrical anhydride thus prepared was added as such to the already swollen resin kept in a flask. The DMAP (1.22 mg, 0.1 equivalents to the loading
efficiency of the resin) was added to the above solution. Shaking was continued for 5 h at room temperature. The resin was washed five times with DMF to remove unreacted components. After coupling of Fmoc-cysteine to resin end-capping of the unreacted functional group on Wang resin was done to avoid truncated peptide fragments in further synthesis. Acetylation mixture was prepared (193 DMF : 6 acetic anhydride : 1 DIEA) and mixed with dried resin and kept in shaker at 37°C for 1 h. Thereafter, beads were washed five times with DMF and deprotection was done to remove N-terminal protection from Fmoc-Cys-Wang resin as given in the next paragraph.

**Removal of N-terminal Protection from Amino Acid Coupled to Resin**

A 20% piperidine in DMF was added to the resin and allowed to stand for 20 min at room temperature with occasional shaking. Piperidine solution was drained off and the resin was washed five times with DMF.

**Coupling of Di-Fmoc-Lys-OH**

The cysteine bound resin was taken in modified Merrifield flask and 177.2 mg of di-Fmoc-lys-OH (2.5 mM) along with 40.5 mg HOBt (2.5 mM was dissolved separately in 5 ml of DMF at 0°C. HBTU, 109.9 mg (2.4 mM) was added to activate the di-Fmoc-lys-OH. This activated di-Fmoc-lys-OH was added to the resin and left for coupling for 2 h at 37°C in a shaker at 160 rpm. Excess reactants were removed by filtration and resin was washed 5 times with DMF. Coupling efficiency was determined as previously described and found to be 90%. At this stage HBTU was added to the end-capping of unreacted amino groups on Cys-Wang resin, this provided a MAP core having two arms.

**Sequential Synthesis of Full Length Peptide on MAP Core**

After determining the coupling efficiency, peptides were synthesized by sequential addition of Fmoc amino acids on the MAP core, as per the sequence in the same manner described previously. The MAP core on 100 mg resin beads was utilized to synthesize each MAP peptide. Once the desired length of the peptide was achieved, the N-terminal end was deprotected and acetylated before cleavage from the resin.

**N-terminal Acetylation of Resin Bound MAP Peptides**

After addition of the last amino acid as per the sequence, deprotection was done using 20% piperidine. Again a mixture of DMF: acetic anhydride : diisopropyl ethylamine (193 : 6 : 1) was added to the resin bound peptide. The mixture is kept at room temperature with constant shaking for 2 h. Excessive reagent was removed and beads were washed thoroughly with DMF and finally dried by washing with dry methanol.

**Side Chain Deprotection and Cleavage of MAP Peptide from Resin**

The acetylated MAP peptides were deep-rooted and cleaved by preparing a cleavage mixture comprising of TFA : thioanisol : water : phenol : EDT (92 : 2 : 2 : 2 : 2, v/v). Cleavage mixture was added (@ 200 μl per tube to the resin bound peptide and left at room temperature for 5 h with continuous shaking at 500 rpm. The tubes were centrifuged at 10,000 rpm for 5 min and TFA extract was transferred to different tubes after labelling them. TFA solution was poured in chilled dry ether and peptides were precipitated. The precipitated MAP peptides were washed 5 times with dry, chilled ether, followed by addition of vacuum drying. The white powder thus obtained was stored in a desiccator for further use.

**Analysis of Peptide Purity Using Reverse Phase-HPLC (RP-HPLC)**

Peptides were purified on semi-preparative RP-HPLC (Ultropac column, TSK ODS, 120T, 10 μm, and 7.8 X 300 mm) with the following gradient elution of water and acetonitrile having 0.1% TFA (v/v) as given in Table 1. The flow rate

### Table 1 — List of peptides synthesized

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Chain</th>
<th>Peptide sequence from N to C terminal</th>
<th>Location</th>
<th>Number of residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>A full</td>
<td>DNYIKMSDKCCNVGCTRRELASRC</td>
<td>1-24</td>
<td>24mer</td>
</tr>
<tr>
<td>P2</td>
<td>B full</td>
<td>TDDKKLKACGRDYVRLLQIEVCGSSWWGRKAGQRLRE</td>
<td>1-35</td>
<td>35mer</td>
</tr>
<tr>
<td>P3</td>
<td>A</td>
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<tr>
<td>P4</td>
<td>A</td>
<td>KMSDKCCNVGCTRRELASRC</td>
<td>5-24</td>
<td>20mer</td>
</tr>
<tr>
<td>P5</td>
<td>A</td>
<td>DKCCNVGCTRRELASR</td>
<td>8-23</td>
<td>16mer</td>
</tr>
<tr>
<td>P6</td>
<td>B</td>
<td>YVRLLQIEVCGSSWWGRKAGQRLRE</td>
<td>13-35</td>
<td>23mer</td>
</tr>
<tr>
<td>P7</td>
<td>B</td>
<td>TDDKKLKACGRDYVRLLQIEVCGS</td>
<td>1-23</td>
<td>23mer</td>
</tr>
</tbody>
</table>
was kept 1.0 ml/min for preparative purifications. Chromatograms were monitored at 220 nm and 280 nm using a photodiode array (PDA) detector. An eluted peptide from semi-preparative HPLC was collected and concentrated in speedvac vacuum concentrator (Eppendorf, USA). The peptides thus purified were used for further study.

**Keyhole Limpet Hemocyanin (KLH) Conjugation of Linear Peptides**

The linear peptides P₁ and P₂ were conjugated to KLH for their subsequent use in immunization for raising hyper-immune serum. A 0.1 M N-hydroxysuccinimide (NHS) and 0.4 M N-ethylcarbodimid (EDC) were prepared, and aliquots of 200 µl were stored in -20°C until further use. A 100 µl of 4.4 mg/ml KLH was also prepared. A 200 µl each of the NHS and EDC were added to 100 µl KLH, and incubated for 30-45 min. Then added 200 µl of peptide 1 and 2 into the above mixture and incubated overnight at room temperature. The conjugated peptides were separated from unconjugated using a 30 kDa cut-off filter (Corning, England) and centrifuged at 5000 rpm for 5 min.

**Expression of Canine Pro-RLX in Recombinant form in Prokaryotic System**

A set of primers were designed having some additional nucleotides added at the 5' ends of the primer for cloning in pHam N-His SUMO vector (ExRLXF3pRham-Sumo 5'-CGCGAACAGATTGGAGGTCTTAAGGCATGTGGTCGTGAT-3', ExRLXR3pRham-Sumo 5'-GTGGCGGCCGCTCTATTAAGCAAGCTCTCTCTGGTGATGAT-3'). The PCR amplification was carried out using the pPICZalpha/Rlx5 plasmid as template. The pPICZalpha/Rlx5 was sequenced earlier and sequence data is available in NCBI GenBank (Accession no KC977307) database. The PCR product thus amplified using Pfu was gel eluted and set for cloning with linearized pHam N-His SUMO vector. The ligation mix was transformed to *Escherichia coli* 10G chemically competent cells. The transformants were screened by colony PCR. The recombinant clones were used for expression studies using SDS-PAGE and Western blotting. The recombinant clone was set for bulk culture and the induced culture was lysed and solubilized using buffer containing guanidine HCl and the rRLX purified using Ni-NTA affinity matrix using gradient pH 6.0 to 4.5.

**Raising of Hyper-immune Sera in Rabbit against the Recombinant Pro-relaxin**

Two male rabbits were immunized with purified recombinant pro-relaxin. Preimmune sera was collected from both the rabbits before immunization. A total amount of 100 µg of antigen was used for each immunization. Equal volume of antigen and Freund’s complete adjuvant (Sigma Aldrich, USA) taken for preparation of antigen and injected subcutaneously at four sites. Three subsequent boosters were given in the same manner at 15 days interval mixed with the same adjuvant. After one week of last injection, blood was collected, serum was separated and tested for the presence of antibodies using ELISA. Serum was aliquoted and preserved at -20°C for further use.

**Raising of Hyper-immune Sera Chicken against Relaxin Peptides**

Two layer chicken were immunized with each peptide. Pre-immune sera were collected from all the birds before immunization. A total amount of 100 µg of peptide antigen was used for each immunization. Equal volumes of synthetic peptide and Montamide ISA adjuvant (IVRI, India) taken for preparation of antigen and injected subcutaneously at four different sites. Three subsequent boosters were given in the same manner at weekly interval. After one week of last injection, blood was collected, serum was separated and titrated for the presence of antibodies using ELISA. Serum was aliquoted and preserved at -20°C for further use.

**Purification of Immunoglobulins**

Immunoglobulins from chicken serum were purified by salting out method using sodium sulfate. One millilitre of serum was diluted in equal volume of distilled water, 0.18 g/ml of sodium sulfate was added gradually with constant stirring at room temperature. Again, this mixture was stirred at room temperature for 30 min and was kept overnight at 4°C. The following day, the mixture was centrifuged at 16000 x g at room temperature for 15 min and the pellet was dissolved in distilled water and the volume was adjusted to the original volume. The resulting solution was once again precipitated with 0.14 g/ml sodium sulfate and pelleted as before, dissolved in distilled water and dialyzed against normal saline (0.154 M NaCl). The purified antibodies were stored at -20°C until further use.
Dot-blot of the Synthetic MAPs and KLH Conjugated Linear Peptides

Dot blot was performed with purified MAP core peptides and KLH conjugated linear peptides for their reactivity to hyper-immune serum raised against the recombinant pro-relaxin already available in the laboratory. The PVDF transfer membrane of 0.2 µm pore size (MDI, India) was cut into appropriate size, soaked in methanol for 30 s and left in transfer buffer (Tris base 118.7 mM, glycine 40 mM, methanol 20%) for 10 min, followed by washing with distilled water and then allowed for drying. The solutions of different peptides (100 µg/ml) were prepared in phosphate buffered saline (PBS) and applied on to a designated spot on the membrane. The membrane was blocked with a 1% bovine serum albumin (BSA) and incubated overnight at 4°C. Following day, the membrane was washed thrice with PBS-T (0.05%) of 5 min each. The membrane was then incubated at 37°C for 1 h with rabbit anti-prorelaxin antibody (1:200 dilutions). Excess antibody was washed thrice with PBS-T (0.05%), followed by incubation at 37°C for 1 h with goat anti-rabbit-HRP conjugate (1:1000 dilution in PBS-T) (Santa Cruz, USA). Excess conjugate was washed thrice with PBS-T (0.05%) and membrane was then immersed in 20 ml of substrate solution (diaminobenzidine 0.03 mM in 50 mM Tris-HCl pH 7.3, 8% nickel chloride 50 µl, hydrogen peroxide 0.01 mM) and allowed for color development.

Indirect Sandwich ELISA

A 96 well flat bottom Maxisorp ELISA plate (Nunc, Denmark) was coated with different chicken anti-Rlx peptide antibodies (1:2500 dilution in coating buffer) and incubated overnight at 4°C. Following day, plate was washed thrice with PBS-T (0.05%), incubated at 37°C on a shaker for 1 h with 3% skim milk powder (HiMedia, India). The known pregnant and non-pregnant sera were added to respective wells and further incubated 37°C on a shaker for 1 h. Subsequently, plate was washed thrice with PBS-T (0.05%) and rabbit anti-pro-relaxin antibody (1:25000 dilution in PBS-T) was added and then incubated at 37°C on a shaker for 1 h. Again plate was washed with PBS-T (0.05%) five times and incubated 37°C for 1 h with goat anti-rabbit HRP conjugate (1:40000 in PBS-T). Excess conjugate was washed with PBS-T (0.05%) five times and 150 µl of substrate was added to each well left at 37°C for 10 min for color development. The reaction was stopped with 50 µl 4N H2SO4 and measured OD at 450 nm with reference reduction at 595 nm.

Results

Totally seven peptides were synthesized using solid phase peptide synthesis chemistry with Wang resin as supporting matrix. Two among them were synthesized in linear format and five peptides in MAP format with two arms. The MAP-core was having lysine mosaic with cysteine at the C-terminal end. The sequence and molecular weight of linear as well as MAP core peptides are listed in the Table 2. In the initial characterization, all the five chemically synthesized peptides in MAP format showed specific reaction with rabbit anti-prorelaxin antibodies. Further, the

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Water (%)</th>
<th>Acetonitrile (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
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<td>100</td>
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</tr>
<tr>
<td>40</td>
<td>STOP</td>
<td></td>
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</tbody>
</table>
two linear peptides conjugated to the KLH also reacted with the rabbit anti-prorelaxin antibodies. However, the unconjugated KLH and the blank spots did show any reaction (Fig. 1).

The cleaved peptides were then subjected to semi-preparative RP-HPLC. Most of the peptides got eluted around 20-26 min at 80-98% acetonitrile gradient with water. The retention time and percent acetonitrile on which peptides eluted is given in Table 3. The recombinant prorelaxin expressed in E. coli 10G cells showed a specific protein band of 32 kDa including the SUMO and histidine fusion tags on SDS-PAGE and Western blotting (Fig. 2a & 2b). Characterization hyper-immune serum raised against the anti-Rlx peptides in chickens using dot-blot coated with the fixed amount of recombinant pro-relaxin showed better reactivity to chicken anti-Rlx P1, P4, P5 and P7 antibodies (Fig. 3).

An indirect ELISA using prorelaxin as coating antibody showed a detection limit of 0.781 – 25.0 ng (R² = 0.992) as shown in Figure 4. The chicken anti-

Rlx P4 antibodies gave better result as compared to the rest of anti-Rlx peptide antibodies in terms of differentiating the pregnant bitches from non-pregnant bitches. Hence, the chicken anti-Rlx P4 has been used as capture antibody and rabbit anti-prorelaxin as detection antibody for further refinement of indirect sandwich ELISA using checker board method with a known pregnant and non-pregnant bitch serum. Thirty five canine serum samples which included 21 non-pregnant, 11 pregnant and 3 male samples were screened using the sandwich ELISA. Among the 11 pregnant serum samples six delivered normally and the rest of the bitches aborted a few days after the serum collection with one or two dead fetus and the sandwich ELISA results are as shown in Figure 5.

**Discussion**

Abortion is often difficult to diagnose as the bitch rarely shows well defined clinical signs (often no signs at all or just a short lasting malaise or anorexia). The canine relaxin (Rlx) radioimmune assay has been fully validated for use in dogs24; and successfully validated and adapted for use in a variety of wildlife species primarily as a noninvasive approach to distinguish pregnant from non-pregnant animals26-29. Though, the Rlx assays are employed for pregnancy diagnosis. The present study was undertaken to

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**Table 3 — Peptide elution conditions**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Retention time (min)</th>
<th>Elution (acetonitrile %)</th>
</tr>
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<tbody>
<tr>
<td>P1</td>
<td>25.5</td>
<td>96</td>
</tr>
<tr>
<td>P2</td>
<td>25</td>
<td>95</td>
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<tr>
<td>P3</td>
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<td>96</td>
</tr>
<tr>
<td>P7</td>
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evaluate Rlx peptide as a marker for the well-being of the fetus in canines.

Seven different peptides containing predicted epitopes of Rlx were designed. The purification\textsuperscript{30} or chemical synthesis\textsuperscript{31–34} of active Rlx hormone has been successfully achieved but its availability in good amount and affordability is still a challenge that needs to be addressed. The advantage of using peptides instead of proteins as antigen for raising antibodies is its availability and ease of producing anti-peptide antibodies against protein isoforms and site specific phosphorylated proteins\textsuperscript{35}. Further, the multiple antigenic peptide (MAP) technology is a novel and versatile strategy designed to overcome the limitations of carrier protein coupling and other methods to improve the immunogenicity of the peptides. Additionally, the reactivity of the MAPs in immunological screening technique is found to be as good as the native antigen and even better than some viral antigens. The MAP provides a macromolecular structure of the small antigenic determinant units and therefore, it can be used successfully to produce anti-peptide antibodies having higher titer against the same peptide conjugated to keyhole limpet haemocyanin (KLH), bovine serum albumin (BSA) or ovalbumin (OVA) as a carrier protein\textsuperscript{36}. Totally seven peptides were synthesized using solid phase peptide synthesis chemistry, two are in linear format and 5 peptides in MAP format with two arms. The P1 and P2 are synthesized in linear format for two reasons, firstly, they represent complete A and B chains of the mature Rlx, respectively. The second reason is that the syntheses of peptides having more than 25 amino acids are very difficult and often leads to synthetic errors. The peptides having more than 90% purity by RP-HPLC was used in further studies.

Before immunization, the specific reactivity of Rlx peptides was checked on the dot-blot using the anti-prorelaxin antibodies available in the laboratory. All the five MAPs and two linear peptides conjugated to KLH showed reactivity with the rabbit anti-prorelaxin. With this confirmation, immunization of chicken with peptides was carried out to raise the hyper-immune sera. As per the standard protocol, the chicken was immunized and hyper-immune serum was collected. In our studies, irrespective of the peptide, the serum anti-Rlx peptide antibody titer was found to be moderate in indirect ELISA where a fixed
amount of recombinant pro-Rlx was coated. Further, the antibody titer was better against peptide P4 and P5 compared to rest of the peptides.

The specificity of the rabbit anti-proRlx was checked for its immunoreactivity to the canine placental extract and serum which showed specific reaction to Rlx (data not shown). All the anti-Rlx peptide antibodies raised in chicken were initially checked for the detection of Rlx in pregnant, non-pregnant and male dogs in indirect sandwich ELISA format using anti-Rlx peptide as capture antibody and rabbit anti-prorelaxin as the detection antibody. The chicken anti-Rlx P4 gave better results in terms of differentiating the pregnant bitches from non-pregnant bitches. Hence, the chicken anti-Rlx P4 has been used as capture antibody and rabbit anti-prorelaxin as detection antibody for further refinement of indirect sandwich ELISA using checker board method with a known pregnant and non-pregnant bitch serum. Due to non-availability of any pregnancy diagnosis kit, the pregnancy status was confirmed by physical examination/ultrasonography and finally by confirming the successful whelping of the bitches. The serum from a full term pregnant bitch nearing the whelping was chosen as a positive control and serum from a non-pregnant bitch as a negative control for standardization of ELISA.

The screening of serum samples of non-pregnant bitches (21 numbers) was carried out using the standardized sandwich ELISA protocol that gave a mean absorbance of 0.153 ± 0.139. The absorbance of non-pregnant sera ranged from 0.006 to 0.335. The calculated cut-off value for pregnant comes around 0.57. Two among 21 non-pregnant serum samples showed the reading above the cut-off. The diagnostic specificity of the assay was found to be 90.47%. Among the true pregnant serum samples (11 numbers) screened 4 showed the absorbance above the cut-off value for pregnancy and successfully delivered healthy puppies. Five bitches which showed the absorbance below the set cut-off value aborted after a few days of blood collection with one or two dead fetuses. Moreover, four bitches (43 d, 62 d, 50 d and 45 d pregnant on the day of blood collection) showed the absorbance above the cut-off value and successfully delivered healthy puppies. The results clearly indicate that the immune reacting Rlx level gets elevated in bitches, where all the fetus are alive. However, pregnant bitches with one or two dead fetuses, the concentration of Rlx falls or remained at the basal level. In agreement with our findings, a decreased concentration of Rlx in the serum has been reported in aborted bitches38-39. The concentration of Rlx also depends on the number and health status of puppies. In conclusion, the Rlx will be a useful marker peptide for monitoring the pregnancy rather for using it for the pregnancy detection and tells about the well-being of the fetus.

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References


