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IDENTIFICATION OF PUTATIVE MUP DURING ESTRUS PERIOD IN FEMALE RAJAPALAYAM BREED DOG BY MALDI-TOF ANALYSIS

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Abstract– A low molecular mass protein (range from 17 to 20 kDa) found in urine, faeces, saliva, glandular secretions, and other pheromone sources has been discovered as a Ligand-carrier (i.e., pheromone carrier) involved in chemical-communication among mammals. The present study examines whether a low molecular protein can be found in the urine of a female Rajapalayam dog and, if so, whether its expression varies during the estrous cycle. SDS-PAGE and MALDI-TOF MS were used to detect estrus-specific urinary proteins in urine samples taken from eight female dogs. The total urinary protein level was substantially greater ($p< 0.05$) in the estrus phase than in the other phases. The results of SDS-PAGE analysis revealed seven different molecular masses of proteins across three phases of estrous cycle, ranging from 13 to 99 kDa. The intensity of the 17 kDa molecular mass protein was significantly higher during estrus phase and incredibly reduced during postestrus, we speculated that it may be an ovulation-indicating protein. The 17 kDa band was digested in-gel with trypsin before being analysed using MALDI-TOF/MS and MASCOT. MALDI-TOF MS and MASCOT searches revealed the presence of MUPs (major urinary proteins) in the 17 kDa band. The present study concludes that MUPs are exclusively expressed in large amounts during the estrus phase. They might transport and prolong the longevity of volatiles in scented sites, thereby providing olfactory cues (i.e. attraction towards males). In addition, the specific expression of MUPs in Rajapalayam dog can be exploited to detect estrus.

INTRODUCTION

It is well documented that vertebrates interact chemically through the use of soluble proteins (pheromone carriers). Chemical sensing and pheromone transport are also facilitated by these proteins. These proteins are released into major body fluids like as urine, faeces, saliva, and specialized scent glands etc., and transport compounds as endogenous ligands with pheromone properties (Achiraman and Archunan, 2002; Ponmanickam and Achunan, 2006). In addition, the proteins in pheromonal sources preserve and transmit the volatile compounds while also play a critical function in chemo-signalling between animals and coordinating animal behaviour. They also serve as pheromone stabilisers in the environment, allowing for the delayed release of odorants by delaying the evaporation or destruction of pheromones, thereby increasing the scent mark's shelf-life (Robertson *et al*., 1993; Flower, 1996). Further, these proteins are remarkably resistant to

drying and heating, and when discharged into the environment, they are unlikely to denature rapidly (Hurst *et al*., 1998).

In the south India, notably in Tamil Nadu, the Rajapalayam breed dog is the most common indigenous breed. This breed have found to be outstanding companion animals, with their best qualities being companionship, house security, wild animal hunting, and cattle herd protection and so on. Some canine breeds have an exceptional sense of smell, which is used to train them for tracking and smelling out criminals (Divya, 2012). So yet, only a few researches have been conducted on the Rajapalayam breed. Further, there is no information on urinary proteins in the Rajapalayam dog's estrous cycle. As a result, the current study was conducted to determine the differences in urine proteins in female Rajapalayam dogs at various estrous cycle phases.

MATERIALS AND METHODS

Animal

Twelve Rajapalayam breed dogs (Rajapalayam breed, *Canis lupus familiaris*; 8 sexually mature female dogs and 4 sexually mature male dogs), were used and ages ranged from 2 to 5 years. The animals were kept in separate rooms on a light-dark cycle of 12 hours L and 12 hours D. This study was conducted at Provokennel, a private Rajapalayam breeds breeding center, as well as at a pet store in Rajapalayam (9°27′0N and 77°33′0E), Virudhunagar District, Tamil Nadu, India. All the studies were carried out under the supervision of a veterinarian who works at the breeding center. This research was performed according to the CPCSEA-IAEC guidelines.

Estrous cycle Determination

A one-month preliminary research was performed with the assistance of a veterinarian to obtain experience with sample collection and behaviour. The anestrous dogs (i.e., non-estrous female dogs) were closely monitored for pro-estrous signs such as vulval swelling and vaginal discharge. Each bitch was allowed to be with a mature stud male for 10 minutes twice daily. The bitch's propensity and response to the stud male were observed. The male was attracted to the bitches when they showed signs of proestrus, but the bitches were not at all receptive. During estrus, the bitch adopts a rigid position, holding her tail to the side, and allows the male to mount her. The day of "heat" or "estrus" was designated as Day "0" of the cycle. Four to fifteen days before estrus the bitches were considered as in proestrus, and five to ten days after estrus the bitches were considered as in postestrus.

Sample collection and Preparation

In order to collect the urine samples and behavioural observation, the dogs (both male and female) were placed in an isolation room, which had its floor cleansed with water. Estrus has been confirmed when a male dog exhibits courtship behaviours toward a female dog and the female is receptive to mounting by the male. The Urine samples were collected from bitches during proestrus, estrus, and postestrus in order to analyze urinary proteins. Urine was collected using a sterile syringe from the clean floor immediately after urination and transferred to 2 mL vials. The urine vials were labelled and transported to the lab in a cold thermos flask, where they were frozen at –20 °C until SDS-PAGE analysis.

A frozen urine sample thawed at room temperature and centrifuged at 12,000 × g for 15 minutes at 4 °C to remove debris and collect the supernatant. The supernatant was segregated with 80% ammonium sulphate, the pellet was recovered with 10 mM Tris, pH 7.2, centrifuged for 20 minutes at 4 °C at 12,000 rpm, and a clear supernatant was obtained. 10 kDa Centricon cut-off membranes were used to concentrate the urine samples. Bradford (1976) method was used to estimate the protein concentration.

Electrophoretic separation of urine sample

All samples were run under reducing conditions in 12% SDS-PAGE (Laemmli, 1970). The 20 µg of protein was loaded onto gel, followed by 4µl of protein standard (medium range molecular weight marker – Genei, Bangalore) to determine the molecular mass. Gels were run for 3 hrs at 50 V, and the proteins were stained with silver stain. Gel documentation (Quantity One, Bio-Rad, CA, USA) was used to determine the intensity and molecular weight of the protein bands in the gels. Pixels were used to measure the band area.

Trypsin In-gel Digestion

Urinary polypeptides were digested with trypsin in gel was conducted as described by Rajkumar *et al.* (2010). The 17 kDa band was excised from the SDS-PAGE gel and destained using 100 µl of 25 mM

NH_4HCO_3 50% (v/v) acetonitrile (1:1) by incubation at 37 °C for 30 min. This process is repeated until the protein band showed no stain. For dehydration, incubating the pooled gel fragments for 20 minutes at room temperature in the dark in 100 µl of 2%-mercaptoethanol/25 mM NH_4HCO_3 was performed after drying with a Speed-Vac. Cysteine alkylation was performed by adding equal amount of 10% 4-vinylpyridine in 25 mM NH_4HCO_3/50 % acetonitrile. As a next step, the pieces were incubated in 1 ml of 25 mM NH_4HCO_3 for 10 min, then dried, and incubated overnight (18 ~ hours) in 25 mM NH_4HCO_3 containing 100 ng of modified trypsin. After a 20 min incubation, the pieces were soaked for 10 minutes in 1 ml of 25 mM NH_4HCO_3 for 10 min, dried, and incubated overnight (18 ~ hours) in 25 mM NH_4HCO_3 containing 100 ng of modified trypsin. Tryptic digests were taken from gels and dried in a Speed-Vac before being frozen at –20 °C for analysis. In the final step, the proteins were resuspended in 10% formic acid immediately before use.

MALDI-TOF/MS and MASCOT Analysis

The tryptic digests were done by mixing equal amounts (2:2) of raw peptide mixture with the matrix solution (α-cyano-4-hydroxycinnamic acid) saturated with 0.1 percent TFA and acetonitrile (1:1), then analysing them in reflectron mode in the positive ion mode with a delay time of 90 ns and accelerating voltage of 25 kV. To boost the signal-to-noise ratio, summations of 300 laser pulses were obtained for each spectrum. External calibration was performed using the peptide I calibration standard, which had masses ranging from 1046-3147 Da. The mass spectra were collected that used a ULTRAFLEX-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) with a 337 nm pulsed nitrogen laser. MS-MS spectra were obtained using an 8 Da window to pick the precursor mass.

The spectra were processed using FLEX analytical software. Monoisotopic peptide masses have been used to search the database. The proteins were identified using the MASCOT database search engine (Matrix Science, London, UK) (http://www.matrixscience.com, search engine). Search results were compared to an estimated population of random matches to calculate probabilistic MW search scores, where p is the absolute likelihood. Scores of greater than 63 were significant in the mascot search ($p<0.05$). Unidentified proteins were found but did not achieve the significance criterion.

RESULTS

The present study showed that the protein concentration significantly varied with urine samples from the female dogs' proestrus, estrus, and postestrus phases. Among them, estrus phased had higher concentration of proteins (63.54±4.39 g/ml) than proestrus (43.16±2.45 g/ml) or postestrus (25.75±1.33 g/ml) phases. The results showed that protein concentrations increased during the estrus period in dogs.

One-dimensional SDS gel electrophoresis was used to compare the protein profile in urine samples at different estrous cycles based on silver staining to characterise the protein elevation. In the SDS-PAGE analysis, seven bands (polypeptides) were observed in the urine samples of three phases of the estrous cycle, whose molecular masses ranged from 13 to 99 kDa, and whose intensities varied during the estrous cycle (Fig. 1). Interestingly, six bands (*viz.* 13, 17, 28, 42, 78, 95kDa) were highly expressed in proestrus and estrus but decreased at the post estrus phase. In addition, the band with 95 kDa showed gradual elevation from proestrus (lower) to estrus (higher, $p<0.05$) and almost disappeared during the postestrus phase. 17 and 28 kDa proteins were most prominent among these polypeptides in all three stages of the urine sample. The present study aimed to identify major low molecular mass proteins in the urine of female dogs. According to the densitometric scanning analysis, the 17 kDa mass

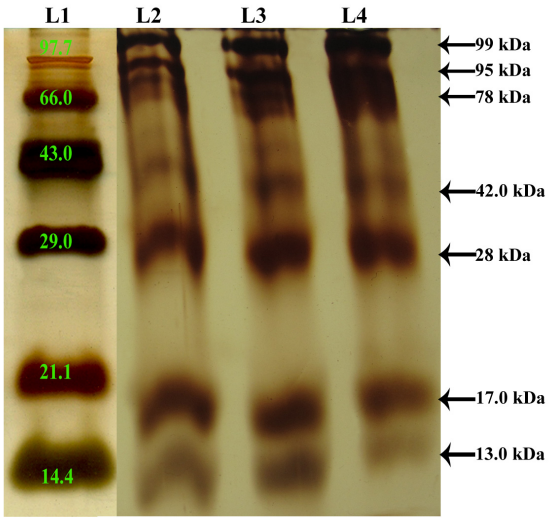

Fig. 1. Electrophoretic distribution of total proteins analyzed by 12% SDS-PAGE.
Lane 1. Medium range molecular weight marker (kDa).
Lane 2, 3 & 4. Urine sample in Proestrus, Estrus and Diestrus respectively.

proteins intensity was pronounced in estrus urine samples as opposed to proestrus and postestrus urine samples (Table 1). Thus, a low molecular mass protein of 17 kDa was selected for investigation by MALDI-TOF analysis.

Table 1. Densitometric pattern of three phases of estrous cycle of female Rajapalayam dogs

Band (kDa)	Estrous cycle (Band Intensity - pixel)		
	Proestrus	Estrus	Diestrus
99	173.58±0.86^{c}	182.17±0.68^{b}	205.25±0.26^{a}
95	162.01±1.19^{b}	199.25±1.05^{a}	163.18±0.25^{b}
78	155.03±0.66^{b}	174.08±0.80^{a}	00.00±0.00
42	15.23±0.28^{c}	172.01±1.19^{a}	19.75±0.33^{b}
28	251.15±0.13^{c}	347.25±0.28^{a}	295.25±0.30^{b}
17	308.15±1.44^{b}	328.05±0.95^{a}	285.31±0.32^{c}
13	185.22±0.38^{b}	304.12±1.03^{a}	175.11±0.56^{c}

Values are articulated in Mean±SE of six experiments. Means with same superscripts are not significant ($p<0.05$) to Duncan Multiple Range Test.

The 17 kDa protein band was excised from the estrus urine and trypsinolyzed for peptide mass fingerprinting. MALDI-TOF was used to get the mass spectrum of the 17 kDa proteins, and MASCOT was used to score and assess the mono-isotopic number of mass spectra. The results showed the presence of "Major urinary protein (MUP)" in the first hit in the 17 kDa protein search list was found, and the score was significant (>50%). In addition, the protein matched with 11 peptides in *Rattus norvegicus* major urinary proteins, representing sequence coverage of 65% (Table 2; Fig. 2). Further, the protein showed matching of 11 peptides with observed masses such as 34-48 (1706.4090 *m*/*z*), 59-74 (1877.5280 *m*/*z*), 59-74 (1893.5310 *m*/*z*), 85-92 (999.0720 *m*/*z*), 93-113 (2396.0070 *m*/*z*), 114-128 (1962.5810 *m*/*z*), 114-128 (1978.5760 *m*/*z*), 119-128 (1312.2100 *m*/*z*), 119-128 (1328.2030 *m*/*z*), 156-164 (1057.1390 *m*/*z*), 165-172 (932.0730 *m*/*z*).

The MASCOT search observation of monoisotopic masses pooled together confirmed that the protein was a major urinary protein (MUP)

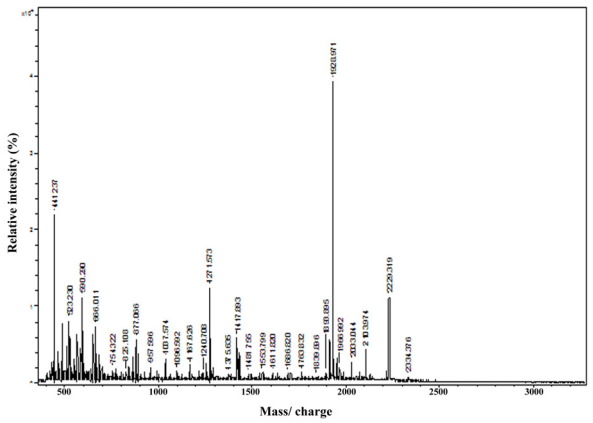

Fig. 2. MALDI-TOF mass spectrum of 17 kDa mas proteins in urine sample of female Rajapalyam dog.

Table 2. Sequence coverage and peptide masses of Major urinary protein (MUP)

[Table shows MUP fragmented with trypsin and map out of total peptides using MALDI-TOF/MS, demonstrating the 'starting' and 'end' of cleavage sites of lysine (K) and arginine (R) with observed and expected masses along with complete peptides residues]

Matched peptides shown in Bold Red

1	MKLLLLLLCL	GLTLVCGHAE	EASSTRGNLD	VAKLNGDWFS	IVVASNKREK
51	IEENGSMRVF	MQHIDVLENS	LGFKFRIKEN	GECRELYLVA	YKTPEDGEYF
101	VEYDGGNTFT	ILKTDYDRYV	MFHLINFKNG	ETFQLMVLYG	RTKDLSSDIK
151	EKFAKLCEAH	GITRDNIIDL	TKTDRCLQAR	G	

Start -End	Observed mass	Expected mass	Sequence
34-48	1706.4090	1705.4017	K.LNGDWFSIVVASNKR.E
59-74	1877.5280	1876.5207	R.VFMQHIDVLENSLGFK.F
59-74	1893.5310	1892.5237	R.VFMQHIDVLENSLGFK.F + Oxidation (M)
85-92	999.0720	998.0647	R.ELYLVAYK.T
93-113	2396.0070	2394.9997	K.TPEDGEYFVEYDGGNTFTILK.T
114-128	1962.5810	1961.5737	K.TDYDRYVMFHLINFK.N
114-128	1978.5760	1977.5687	K.TDYDRYVMFHLINFK.N + Oxidation (M)
119-128	1312.2100	1311.2027	R.YVMFHLINFK.N
119-128	1328.2030	1327.1957	R.YVMFHLINFK.N + Oxidation (M)
156-164	1057.1390	1056.1317	K.LCEAHGITR.D
165-172	932.0730	931.0657	R.DNIIDLTK.T

of 17 kDa. Although no complete amino acid sequence was obtained from the major urinary protein of the Rajapalayam dog, the findings indicated that the identified protein from this species was an unreported major urinary protein.

DISCUSSION

Low molecular mass proteins found in pheromonal sources have been discovered as ligand carriers for pheromonal communication pathways in a variety of animals (Briand *et al*., 2000; Cavaggioni and Mucignat-Caretta, 2000; Pelosi, 2001). In the present investigation, total protein concentration was significantly higher in the estrus urine sample than in proestrus and postestrus. Different molecular mass proteins ranging from 13 to 99 kDa were found in urine samples after electrophoretic separation. Among these fractions, the 17 kDa protein intensity showed modestly during proestrus, peaked during estrus, and then declined dramatically during postestrus. The findings suggest that a higher level of 17 kDa protein intensity in the estrus phase corresponds to a high rate of hormone release. Similar results reported that the urinary protein concentration was increased during the estrus urine sample of female rats (Muthukumar *et al*., 2013).

The MADI-TOF/MS analysis showed that the low molecular mass protein 17 kDa was the major urinary proteins (MUPs) in this investigation. Previous studies of MUPs is belongs to lipocalin family members functioning as pheromones in adult male rats and mice support the existence of significant Major urinary proteins in this study (Cavaggioni and Mucignat-Caretta, 2000; Stopka *et al*., 2007). It is reported that the MUPs found in the urine of mice (*Mus musculus*) function as the hydrophobic ligands for transportation and binding for the volatile pheromones (Novotny, 2003; Thoß *et al*., 2016). These proteins are highly polymorphic (individual variation is significant) and offer different olfactory cues that mediate personal recognition, genetic kin identification, inbreeding avoidance, and other functions (Thom *et al*., 2008; Thoß *et al*., 2016).

According to Cavaggioni *et al.* (1990), Lipocalins are extracellular proteins with a molecular mass of around 21 kDa that have a high selectivity and affinity for tiny hydrophobic compounds. Lipocalins contain a conserved tertiary structure that includes an anti-parallel beta-barrel, an alpha helix, and the lipocalin family signature conserved sequence modif of "GxW" (Flower, 1996; Flower *et al*., 2000). Additionally, the conserved tryptophan (W) functions in ligand affinity, structure, and stability (Gasymov *et al*., 1999). Significant urine protein was the first hit from a MASCOT search, as seen in Table 2. This first hit also includes the GxW pattern, which is a lipocalin signature. The identified protein belongs to the lipocalin family of proteins, according to the findings.

Lipocalin proteins are pheromone carriers reported in a variety of mammals (Beynon and Hurst, 2003; Stopkova *et al*., 2021). For examples, (1) Aphrodisin is a lipocalin protein that is extensively discharged from the hamster's vaginal fluid to facilitate the male's courtship behaviour (Briand *et al*., 2000); (2) Salivary lipocalins is released by the submaxillary glands of mature male boars, which include endogenous ligands 5–alpha–androst–16–en–3–one and 5–alpha–androst–16–en–3–alpha–nol to aid female attraction (Marchese *et al*., 1998); (3) Apolipoprotein-D (ApoD) is secreted in greater quantities in adult male human apocrine sweat glands, which contain the powerful odour 3-methyl-2-heptanoic acid, a constituent able to influence the menstrual cycle (Zeng *et al*., 1996); (4) Alpha 2u globulin is a lipocaline protein, that possesses bonded form pheromones like farnesol 1 and 2, this has been identified in the urine and preputial gland secretion of male rats, it could be used as a female attractant (Ponmanickam and Archunan, 2006; Rajkumar *et al*., 2010); (5) Horse sweat contains the lipocalin EquC1 protein, which carries the ligand oleamide, which serves as a pheromone (D'Innocenzo *et al*., 2006); (6) The volatile compounds 2-sec-butyl-4,5-dihydrothiazol, 3-4-dehydro-Exo-brevicomin, and farnesenes (α and β) are bound in MUPs (major urinary proteins) in male mice, and they are slowly released into the air as the urine begins to dry out, which could accelerate puberty in female mice (Mucignat–Caretta and Cavaggioni, 1995; Zidek *et al*., 1999). The alpha 2u-globulin protein is identified in the post of the preorbital gland in Indian Blackbucks, and it is considered to carry volatile compounds (such as 2-methyl propanoic acid, 2-methyl-4-heptanone, 2,7-dimethyl-1-octanol, and 1,15-pentadecanediol) that are released into the environment through scent marking behaviour, these volatile compounds can be used to defend territory and suppress subordinate behaviours (Rajagopal *et al*., 2015). According to all of these investigations to mention about the Lipocalins play an important role in the ability to bind and transport a wide range of

lipophilic molecules (i.e., volatile compounds) in order to facilitate chemical communication among animals.

CONCLUSION

This is the first direct demonstration of the difference in MUPs in the urine of female dogs during the various stages of the estrous cycle. According to SDS-PAGE analysis, 17 kDa proteins found in the urine predominately during the estrus phases than compare to that of other phases. MALDI-TOF/MS and MASCOT analysis further verified that the 17 kDa protein is characterized as MUPs and belongs to the Lipocalin family. Since MUPs have been identified as a pheromone-carrying protein in rats and mice, suggesting that this protein may play a similar role in female Rajapalayam dogs. It was interestingly to note that the dog is not sedentary and is quite active, racing along the living site and executing regular scent marking behaviour (i.e., urine spray) on the patrolling side. During heat period, the female releasing the sex attractive pheromones (volatile compounds) with higher amount, which behave as chemical love letters to invite the potent mate for successful reproduction. The findings of this study offer novel methods for determining the ovulatory phase in female Rajapalayam dogs by measuring MUPs levels during the estrous cycle. Further, the present study found that the female dogs regulate the expression of specific MUP to coordinate a chemosignal function independently of a pheromone.

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Conflict of Interest

The authors declared that they have no conflict of interest.

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