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# Validation of a newly generated oxytocin antibody for enzyme-linked immunosorbent assays

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**ABSTRACT.** The biological and psychological significance of oxytocin is increasingly recognized; however, reliable assays of oxytocin in biological samples have not been developed. We raised a new oxytocin polyclonal rabbit antibody against synthetic oxytocin. The affinity of antibodies to oxytocin was examined by a radio-immunoassay and compared with that of a previously validated antibody. One antibody showed affinity for oxytocin in the radio-immunoassay. We developed a solid-phase ELISA for oxytocin using this antibody and compared it with existing methods. The newly developed ELISA showed comparable results using urine samples but not using serum samples. These results indicate that the new ELISA is useful for urinary oxytocin; further modifications, such as different extraction methods, are needed for its application to serum oxytocin.

**KEY WORDS:** enzyme-linked immunosorbent assay, oxytocin, polyclonal antibody

Oxytocin (OT) is a nonapeptide hormone that regulates conductance in the uterus and milk ejaculation. In addition to its physiological roles, recent studies have investigated the roles of oxytocin in social behavior and psychology in animals and humans [3]. Several approaches have been used to determine the function of OT in the central nervous system. For example, behavioral changes are induced by the intranasal administration of exogenous OT, which penetrates the central nervous system [1]; however, there is considerable variation in the effects of intranasal OT, and the effect is likely to be weak and undetectable, requiring large sample sizes. Moreover, associations between the concentration of endogenous OT in peripheral fluids (i.e., the serum, saliva, and urine) and behavioral phenotypes have been evaluated [2]. These experiments are based on the finding that endogenous OT in biological fluids of the periphery can be an index of OT activity in the brain [13]. In fact, magnocellular OT neurons in the paraventricular hypothalamus projecting to the posterior pituitary gland and emitting OT in the periphery influence social behavior [14]. Despite the importance of precise measurements of peripheral OT, the reliability of assays is still under debate [6]. Indeed, previous studies have reported low correlations among values obtained using different methods, fluid types, and sample types (e.g., crude and extracted).

Using a radio-immunoassay (RIA), we have previously found correlations between urinary and blood OT concentrations in dogs and humans [8, 10]. In these studies, an antibody was raised in rabbits by Higuchi *et al.* and showed high titers and selectivity [4]. However, the antibody was polyclonal, and the amount was limited. Hence, it is necessary to raise a new antibody and to establish the validity of the OT assay system: In this study, we aimed to characterize the reliability of a newly generated OT antibody to gain insight into its validity as a marker for the physiological and behavioral effects of OT. We raised OT polyclonal antibodies following the previously described protocol and validated the reliability of the antibodies by ELISA. We followed a previously described protocol in which rabbits were immunized for 6 months [4]. Immunization and purification were conducted by Protein Purify Co., Ltd. (Isezaki, Japan). In brief, synthetic oxytocin (Peptide Laboratory, Co., Ltd., Osaka, Japan) was conjugated with bovine serum albumin (BSA), and 0.15 mg of OT was injected subcutaneously with Freund's complete adjuvant, 17 times at 2-week intervals in three rabbits. Blood was collected via the ear lobe and plasma protein levels were measured. After

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immunization, whole blood was collected under deep anesthesia (5% isoflurane) and stored at −20°C. The final concentrations of plasma proteins were 0.294 mg/ml (#1), 0.314 mg/ml (#2), and 0.372 mg/ml (#3).

Binding affinity was evaluated by RIA. Antibodies were extracted by affinity chromatography purification of polyclonal IgG. Standard OT was incubated with the extracted antibody (dilution × 12,800) for 24 hr at 4°C. OT labeled with $I^{125}$ was added and incubated for 2 days. Aliquots were incubated with the secondary antibody and polyethylene glycol (PEG) and centrifuged, and the centrifugal supernatant was discarded. Radioactivity was measured. The previous antibody (Higuchi antibody; HAB) showed a dose-dependent decline of $I^{125}$-OT (Supplementary Fig. 1). The newly generated antibody #1 had a binding affinity to $I^{125}$-OT and showed a decline with an increase in standard; however, the measurement range was different from that of HAB. The other newly generated antibodies (#2 and #3) had lower binding affinities to $I^{125}$-OT; therefore, antibody #1 was used in subsequent analyses.

*Selectivity test*: As OT has a similar protein composition to that of vasopressin, the specificity of the antibody was tested by western blotting. As OT and vasopressin are small molecules, the peptides were fused to glutathione *S*-transferase (GST) and expressed in *Escherichia coli*. GST, GST-OT, and GST-vasopressin were detected by an anti-GST antibody (Supplementary Fig. 2, lanes 1, 2, and 3 in the lower panel). As expected, the anti-OT antibody recognized only GST-OT and not GST or GST-vasopressin (Supplementary Fig. 2 upper panel). To obtain quantitative differences in OT and vasopressin, we performed a densitometric analysis of the exposed blots. The relative amount of vasopressin was less than 0.1% of the amount of OT (Supplementary Fig. 2). These results indicate that the anti-OT antibody has high specificity for OT.

*Establishment of an ELISA*: We validated a solid-phase ELISA using the newly characterized antibody. The procedure was as follows:

1. OT was solidified. Briefly, 100 µl of 5 ng/ml OT was added to each well and incubated for 24 hr at 4°C.
2. For blocking, after washing the wells, 1% Tween20 and 5% skim milk in 0.1 M phosphate-buffered saline (PBS) were incubated for 1 hr at room temperature (RT).
3. Standard OT was prepared by diluting 1.95 to 2,000 pg/ml standard OT in phsophate buffered saline with Tween-20 (PBST).
4. For sample preparation, urine samples from dogs were diluted with distilled water (×20). Plasma samples were incubated with 1% sodium dodecyl sulphate (SDS) at 98°C for 10 min. After boiling, the samples were placed at RT and then fractionated by 3,000 Da molecular weight cutoff using a Vivaspin ultrafiltration column (Sartorius Stedim Lab Ltd., Stonehouse, UK).
5. A volume of 50 µl of samples and standard OT was incubated with 50 µl of the OT antibody (#1, × 14,000) overnight at 4°C.
6. After rinsing by washing buffer, 100 µl of the secondary antibody (Anti-Rabbit IgG, HRP-Linked Whole Ab Donkey NA934; Cytiva, Co., Ltd., Marlborough, MA, USA) in PBST (×3,000) was incubated for 1 hr at RT.
7. After rinsing by washing buffer, 100 µl of TMB solution (5120-0053, Sera Care, Milford, MA, USA) was added to each well and incubated for 10 min, and 100 µl of stop solution was added. Color development was measured at 450 nm using a plate reader (iMark microplate reader; Bio-Rad Co., Ltd., Hercules, CA, USA).

The obtained standard curve is shown in Supplementary Fig. 3.

*Dilution and Spike test*: All animal experiments were approved by the Animal Ethics Committee of Azabu University (#190927-1). A 2× dilution of dog urine and 8× dilutions of urine samples with known concentrations of OT standards were used for dilution and spike tests, respectively. The estimated and actual concentrations were compared. For both tests, there was a significant correlation between the estimated and the actual values (Fig. 1).

*Comparison with other methods*: Finally, we compared the measurement results to those obtained by existing methods [11]. For urine samples, the standard method utilizes a commercially available ELISA kit (ADI-900-153A; ENZO Life Sciences, Inc., Farmingdale, NY, USA). The ENZO kit was used following the manufacturer's protocol, and the correlation between the results obtained by the new method and the previously established method [11] was determined (Fig. 2A). Plasma samples were extracted

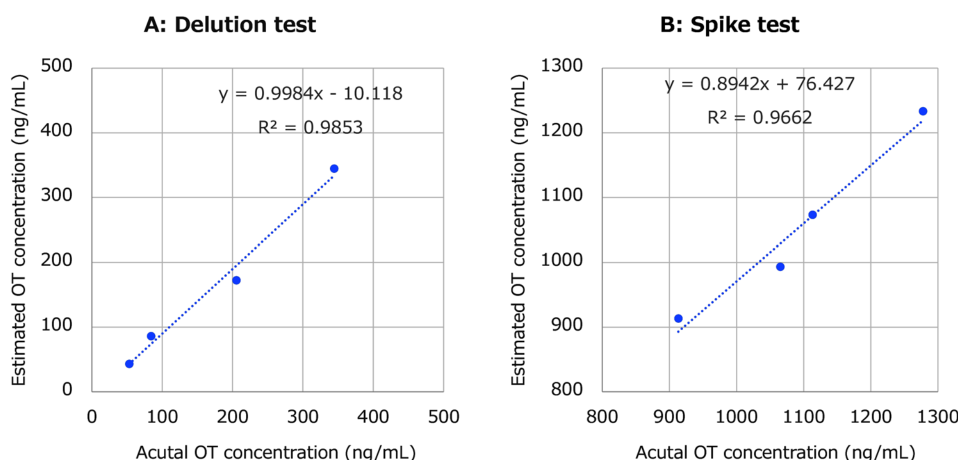


**Fig. 1.** Dilution test (A) and spike test (B) of the new solid phase ELISA of oxytocin (OT). In both tests, there were strong correlations (dilution: $y$=0.9984$x$ −10.118, $R^2$=0.9853. Spike: $y$=0.8942$x$ + 76.427, $R^2$=0.9662).

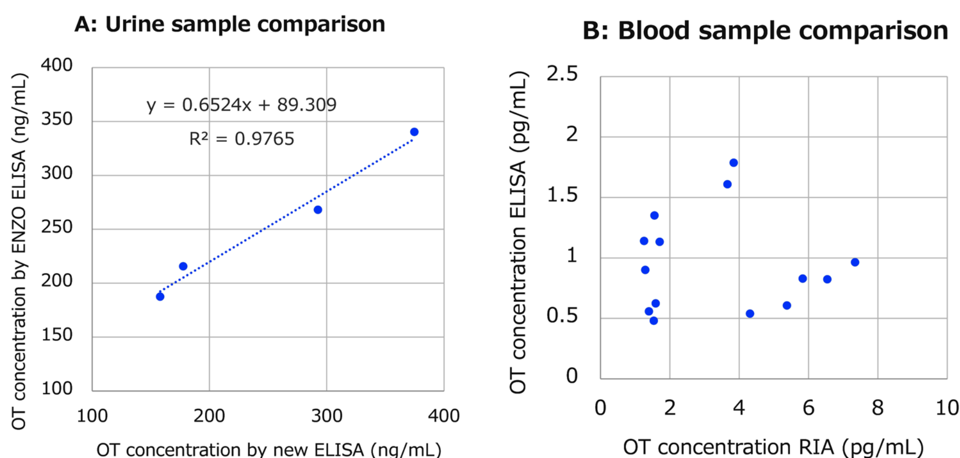


**Fig. 2.** Comparison of concentrations in urine samples (A) and plasma samples (B) determined by the new solid-phase ELISA and established assays (ELISA for urine, radio-immunoassay (RIA) for plasma). In urine samples, but not in plasma samples, there were high correlations ($y$=0.6524$x$ + 89.309, $R^2$=0.9765).

as previously reported and measured by the procedure described above. However, the correlation between the results obtained by the two methods was low (Fig. 2B).

We developed new antibodies to OT, one of which bound to OT with high affinity, comparable to the previously established OT antibody for RIA. The slope of the dose-response curve was low, indicating that the specificity of the antibody was not as high as that of the previous antibody. However, the new antibody is useful for assays of OT in biological samples.

OT measurements in urine samples by the newly developed solid-phase ELISA were highly reliable; dilution and spike test results showed high correlations with estimated values. A comparison with the commercially available kit yielded similar values. The measurable range was 10 to 2,000 pg/ml, which was slightly wider than that of the ENZO kit (15–1,000 pg/ml).

However, the use of plasma samples for OT analyses has important limitations. First, measuring OT in blood samples without extraction is potentially erroneous, given the high risk of contamination with immunoreactive products other than OT [12]. These non-OT immunoreactive products might constitute highly stable plasma housekeeping proteins, which mask the true variation in OT concentrations [5]. In the RIA, extraction was performed with acetone-diethyl ether, as described previously. The RIA for OT is a sensitive method for quantification and is considered the gold standard [7]. For ELISA, we used SDS for extraction. This method showed good dilution and spike test results (data not shown). In the future, other extraction methods, such as using acetone-diethyl ether and sep18-column extraction, must be evaluated for plasma assays.

Another biological sample type for OT assays is saliva. However, a recent study has shown that saliva samples are still problematic [7]. First, there was no correlation between plasma and saliva OT concentrations. Second, concentrations of OT in the plasma and saliva were not correlated at baseline or after the administration of exogenous OT by intranasal or intravenous routs [9]. We obtained similar results for dogs; exogenous OT increased OT concentrations in the urine and plasma but not in the saliva (Supplementary Fig. 4). These data suggest that salivary OT is a weak surrogate measure for peripheral blood levels.

In summary, we generated a new OT antibody and demonstrated that urine OT concentrations can be reliably measured by a solid-phase ELISA using this antibody. Future examinations are needed to adapt the assay to plasma and saliva samples.

CONFLICT OF INTEREST. All authors declare that there are no conflicts of interest.

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