Determination of carcinoembryonic antigen and cancer antigen values with the radioimmunoassay method in healthy females dogs

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ABSTRACT: The aim of this study was to determine reference values of carcinoembryonic antigen and cancer antigen in 32 clinically healthy bitches. The average age of the bitches in each group was as follows: small breeds 3.50 ± 2.30, medium breeds 3.83 ± 3.21, large breeds 6.00 ± 3.22 and giant breeds 2.40 ± 2.43. The average weight in each group was as follows: 1st group 7.94 kg ± 1.84, 2nd group 22.38 kg ± 2.77, 3rd group 35.94 kg ± 7.16, and 4th group 52.75 kg ± 5.04. The cancer markers were determined using human kits. The mean values of the carcinoembryotic antigen markers ± SD were as follows: 1st group 0.18 ± 0.03, 2nd group 0.20 ± 0.03, 3rd group 0.22 ± 0.01, 4th group 0.18 ± 0.04. The statistical significance for the carcinoembryonic antigen markers was $P = 0.0042^{**}$. The values of cancer antigen markers ± SD were: 4.90 ± 1.04, 4.80 ± 1.13, 5.90 ± 1.22, and 4.72 ± 0.97, respectively. The cancer antigen values were statistically insignificant ($P = 0.1762$). Based on obtained values of the mean 95%, we expect a standard for carcinoembryonic antigen of 0.00–0.23 ng/ml and for cancer antigen 0.0–7.00 IU/ml. The results of the present study show that it is possible to use human kits for the determination of carcinoembryonic antigen and cancer antigen in clinically healthy bitches using the radioimmunoassay method.

Keywords: canine; mammary gland; CEA; CA 15-3; tumor markers

List of abbreviations

CEA = carcinoembryonic antigen, **CA 15-3** = cancer antigen

Cancers in human medicine, like in veterinary medicine, have a tendency to grow and are a result of various external and internal factors (MacEwen 1990). Mammary gland tumours have serious effects on female health (Winer et al. 2001), and are the most common type of oncological disease to affect women between the ages of 40–45 (Bland et al. 2005). Mammary gland tumours are the second most common neoplasia found in dogs (Capik et al. 2008), and constitute 42% of all tumours found in bitches. (Brodey et al. 1983).

The most common type of mammary gland tumours in women histologically are carcinomas, and more specifically, invasive ductal carcinomas (Akiyama and Horii 2009). In bitches, mammary gland tumours are malignant or benign, and originate from various types of tissue in the breast (epithelial or glandular tissue, mesenchymal or interstitial tis-

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sue). The majority of those presented are classified as epithelial tumour carcinomas (Misdorp 1999).

Mammary gland tumours in bitches most commonly occur between 8–10 years of age. Breeds with a higher predisposition include the Poodle, English Cocker spaniel, English Setter, Dachshunds, and some terriers. Breeds with a lower predisposition are the Boxer, Chihuahua, Beagle, and some hounds (Kitchel and Loard 1997). Approximately 65% of all mammary gland tumours are observed in the caudal pair of glands. Risk factors for tumour formation are exogenous sex hormones. Other risk factors include repeated pseudopregnancy, and mastopathy (Hahn et al. 1992). The incidence of mammary gland tumours is more common in intact bitches (Benjamin et al. 1999). Although modern technology and radiological screening procedures exist, more practical and sensitive laboratory methods, which can help to detect various neoplasias and provide quantitative assessment regarding growth, invasiveness, metastasis, and therapy are desirable (Muthuswamy and Raste 2000). In veterinary medicine, until now, initial clinical examination – adspection and palpation of lesion, TNM classification of tumours (modified by Owen 1980), followed by fine-needle aspiration cytology which confirmed or disproved malignancy served for evaluation of mammary gland tumuors. Before surgery, biochemical and haematological examinations are performed. After surgical extirpation, the type of tumour is identified histologically according to the WHO guidelines (Misdorp et al. 1999).

In women, the standard diagnostic procedure for breast tumours involves the determination of tumour markers using radioindicative methods. Tumour markers are substances produced by tumours which are capable of infiltrating body fluids. Their concentration in blood serum and plasma is determined by various immunochemical methods including RIA (radioimmunological methods). Only small amounts of tumour markers remain in tumour tissue where they can be detected by immunohistochemical methods or their concentration in tissue cytosols can be measured (Kausitz et al. 2003). The parameters of basic tumour markers are in human medicine presented as CEA (carcinoembryonic antigen) and CA 15-3 (cancer antigen). CEA was one of the first tumour markers identified and described (Sikorska et al. 1988). Some studies suggest that positive CEA values found in serum at the time when a primary breast tumour is diagnosed represent negative prognostic factors (Molina et al. 1998). Recent studies discourage the routine use of the CEA assay because of its low sensitivity in both early and advanced disease compared with CA 15-3 (Fiorella et al. 2001). High values of CA 15-3 can be connected with severely affected tissue and a poor prognosis. Molina et al. (2003) suggested that women with a high concentration of CA 15-3 in blood serum have a worse prognosis, and showed that the CA 15-3 antigen can be the first marker in relapses, as well a reliable prognostic indicator of breast tumour patients (Berruti et al. 1994).

In veterinary medicine, many human diagnostic procedures are used, such as biochemical and haematological tests, or determination of hormone concentrations using human kits.

In human medicine, for the determination of breast tumours, the basic markers CEA and CA 15-3 are used. In veterinary medicine, there is no information about the determination of the tumour markers CEA and CA 15-3 by use of RIA methods.

The aim of the preliminary study:

(a) to verify the ability to determine tumour markers in bitches by use of human kits and to modify procedures for the determination of these markers,

(b) to establish values of the tumour markers CEA and CA 15-3 in clinically healthy bitches.

MATERIAL AND METHODS

Animals

The group of animals examined consisted of 32 clinically healthy bitches from the age of 10 months to 11 years, weighing from 5 kg to 57.5 kg, without evident clinical changes of the mammary gland. The group of dogs was divided into four groups: small breeds 5–10 kg, medium breeds 10–25 kg, large breeds 25–45 kg, and giant breeds 45 kg and more. The average values of each group and mean results are listed in Table 1. The data of each animal consisted of their signalment, medical history, and actual clinical state. The data of all examined animals together with the measured values of CEA and CA 15-3 markers are listed in Table 2.

Clinical examination

The preliminary examination consisted of measuring body temperature, respiratory rate, and femo-

ral pulse rate. The bitch was then weighed. After the weight was recorded, the bitch was examined systematically by way of topographical adspection and palpation of the head, neck, trunk, extremities, as well auscultation of the thorax. Palpation of the abdomen followed, in which all mammary gland units and regional lymph nodes were examined. Following the clinical examination, venous blood from the *v. cephalica antebrachii* or the *v. saphena medialis* (*lateralis*) was taken. A portion of the blood serum was biochemically and haematologically tested. The remaining blood serum was stored at –18 °C until the tumour markers were determined. The transport of the blood serum to the laboratory was carried out in a portable cooler where the temperature was kept constant until arrival.

Determination of tumour markers CEA and CA 15-3

Tumour markers were determined using a radioindicative method – immunoradiometric analysis (IRMA) with the use of commercially available kits for human medicine. Both determinations (CEA and CA 15-3) are based on the sandwich method using two monoclonal antibodies against two different epitopes, which work independently of each other. These markers are not currently available in veterinary medicine. Therefore, a comparison of reagent composition, commercial kits, and commonly used human and veterinary diagnostic sets was performed (Catalog of diagnostic sets). In the comparison, minimal variations were detected during the incubation period, in dilution of samples and pipetted volumes, and therefore it was necessary to optimise the reagents and steps for determination so that concentrations of the markers could be accurately measured from calibration curves. For the determination of CEA markers, we used kits from Beckman-Coulter, Inc. (Prague), and for the determination of CA 15-3 markers, we used kits from DiaSorin (DiaSorin S.p.A, Italy). The obtained data were calculated on machines from Backman Coulter-Immunotech-LB 2111 (multicrystal gamma counter), with software from LBIS (Backman Coulter, Bratislava, Slovak Republic). Values of CEA markers were measured in ng/ml and values of CA 15-3 markers in IU/ml.

In human medicine, the standard values for CEA markers are up to 5 ng/ml and for CA 15-3 markers, the norm is 30 IU/ml with an upper limit of 35 IU/ml.

Modified determination of CEA markers

Prior to determination, all reagents were brought to laboratory temperature and thoroughly mixed. The contents of laboratory bottles containing lyophilized reagents, apart from the samples, were diluted in redistilled water whose volume was marked by labels. The cleaning solution was prepared by dilution with 950 ml of redistilled water. Based on the analysis of various samples, the optimum dilution ratio was determined to be 20 µl of sample to 2000 µl of redistilled water.

Determination procedure

In antibody-coated test tubes, 50 µl of sample and 200 µl of a radioindicator were mixed. After 2 h of incubation at laboratory temperature, and constant mixing (at > 280 vibrations/min), the contents were carefully removed and twice flushed with 2 ml of cleaning solution.

The binding activity was measured using a gamma counter over the course of 2 min.

The calibration curve intervals for measurement ranged from 0.50 to 325.0 ng/ml. The margin of error of the diagnostic kits as stated by the manufacturer was 0.20 ng/ml.

Modified determination of CA 15-3 markers

As in the determination of markers, all reagents for the determination of CA 15-3 markers were brought to laboratory temperature and thoroughly mixed. Lyophilized reagents were reconstituted with redistilled water according to the manufacturer's guidelines. We used various dilutions of blood serum samples. The samples were diluted with redistilled water at a ratio of 20 µl of serum to 2000 µl of redistilled water. From this dilution, another dilution was performed at a ratio of 20 µl of solution to 2000 µl of redistilled water (double-dilution process).

Determination procedure

In antibody-coated test tubes, 100 µl of double-dilution sample and 100 µl of a radioindicator were mixed.

After 2 h of incubation at laboratory temperature and constant mixing (at > 280 vibrations/min) the

contents were carefully removed and triple-flushed with 2 ml of cleaning solution.

Binding activity was measured using a gamma counter over the course of 2 min. The samples of clinically healthy dogs were tested three times.

The calibration curve intervals for measurement ranged from 6.25 to 300.0 IU/ml. The margin of error of the diagnostic kits as stated by the manufacturer was 2.0 IU/ml.

Quality control

Both diagnostic kits were subjected to internal quality control in the laboratory, measured values CV% and proportion of controlled materials was 6.8% (1.62–2.00 ng/ml) for CEA markers and 4.7% (17.7–19.5 UI/ml) for CA 15-3 markers. The laboratory has been subjected to external quality assessment by SEKK-Pardubice from 2005 for CEA markers.

Statistical analysis

The statistical analysis of measured values was performed according to the one-way ANOVA test. The Kruskal-Wallis test was used for analysis of clinically healthy bitch weight and CEA marker values. CA 15-3 markers were statistically analysed using the one-way ANOVA variance test. We compared the CEA markers and CA 15-3 markers with theoretical human values according to the paired t-test.

RESULTS

The mean age of clinically healthy bitches, mean weight, mean ± SD of CEA markers, upper 95% (percentile) in each group as well as mean CA 15-3 marker values and upper 95% for each weight group are listed in Table 1.

The measured values of CEA marker antigen varied between 0.12 ng/ml and 0.24 ng/ml (Table 2). The p value of CEA markers was statistically significant ($P = 0.0042^{**}$) (Figure 1). Measured concentrations of CA 15-3 antigen ranged between 3.02 IU/ml and 7.70 IU/ml (Table 2). The value 7.70 IU/ml occurred only in one case where the bitch was presented shortly after her heat cycle. Because this value may have been influenced by hormones, it was not considered decisive for the upper border of this antigen. The p value for CA 15-3 was not statistically significant ($P = 0.1762$) (Figure 2).

In comparing our results with human reference values of CEA and CA 15-3 markers, there was a statistical significance of $P < 0.0001$ determined in both cases. Based on the measured values of the upper 95%, we expect the following norms: for antigen CEA: 0.0–0.23 ng/ml and for antigen CA 15-3: 0.0–7.00 IU/ml.

DISCUSSION

It generally holds true that many diagnostic procedures and data from human medicine are applicable in veterinary medicine.

The determination of tumour markers in human medicine is considered as a routine procedure for diagnostics and in particular for monitoring oncological diseases.

The CEA and CA 15-3 markers are the most important tumour markers used in breast tumour diagnosis (Ebeling et al. 2002). Generally said, obtained tumour markers from patients with breast tumours cannot be used for the primary diagnosis of disease because of low specificity and sensitivity (Lamerz et al. 1993). Their use in early metastasis detection looks promising and is widely accepted (Safi et al. 1991). Many studies have tried to evaluate the prognostic features of these markers (some by analysing serum, others by analysing tissue), but many of these studies analysed low numbers of patients or used short time periods for determination,

Table 1. Average values of compared bitches groups

Group	Mean age (year ± SD)	Mean weight (kg ± SD)	Average CEA (ng/ml ± SD)	Upper 95% for CEA (ng/ml)	Average CA 15-3 (IU/ml ± SD)	Upper 95% for CA 15-3 (IU/ml)
1	3.50 ± 2.30	7.94 ± 1.84	0.18 ± 0.03	0.21	4.90 ± 1.04	5.77
2	3.83 ± 3.21	22.38 ± 2.77	0.20 ± 0.03	0.22	4.80 ± 1.13	5.83
3	6.00 ± 3.22	35.94 ± 7.16	0.22 ± 0.01	0.23	5.90 ± 1.22	6.87
4	2.40 ± 2.43	52.75 ± 5.04	0.18 ± 0.04	0.22	4.72 ± 0.97	5.53

Table 2. Signalment and results of determined markers in clinically healthy bitches

Number	Breed	Age (years)	Weight (kg)	CEA (ng/ml)	CA 15-3 (IU/ml)
Group 1 (small breeds; *n* = 8)					
	Yorkshire Terrier	4	5	0.19	4.74
	Jack Russell Terrier	3	6.5	0.20	3.10
	Poodle	7	7	0.14	5.65
	Mixed-breed	2	7.5	0.12	5.28
	West Highland White Terrier	4	8	0.22	5.93
	West Highland White Terrier	6	9	0.20	3.95
	Mixed-breed	1.5	10	0.19	6.10
	Beagle	0.8	10.5	0.16	4.47
Group 2 (medium breeds; *n* = 8)					
	American Staffordshire Terrier	4	18	0.21	5.80
	German Shepard	0.8	20	0.13	3.56
	German Shepard	1	20	0.22	6.50
	Mixed-breed	5	22	0.22	4.70
	Hungarian Vizsla	3	24	0.19	5.09
	Dobermann	6	25	0.20	3.26
	German Shepard	1.5	25	0.21	5.76
	American Staffordshire Terrier	10	25	0.18	4.37
Group 3 (large breeds; *n* = 8)					
	Dobermann	5	26	0.22	7.70
	Boxer	3	28	0.23	4.18
	Dobermann	8	30	0.21	6.64
	German Shepard	4.5	35	0.22	4.68
	Rottweiler	3	40	0.23	6.79
	Rottweiler Mix	3.5	42	0.22	4.75
	Labrador Retriever	11	42.5	0.22	6.20
	Giant Schnauzer	10	44	0.24	5.84
Group 4 (giant breeds; *n* = 8)					
	Tibetan Mastiff	2	45	0.20	3.02
	Great Dane	0.9	46	0.11	4.02
	Rottweiler	2	50	0.20	5.60
	Golden Retriever	8	55	0.13	4.10
	Alaskan Malamute	3	55	0.23	5.13
	Moscow Watchdog	1	57	0.21	6.02
	St. Bernard	1	56.5	0.19	4.67
	Irish Wolfhound	2	57.5	0.18	5.16

and employed only one-way analysis (O'Hanlon et al. 1995).

The cut-off values depend on many factors – for example, the antibody used, concentration, determination methods, and other analytical characteristics of the methods. The choice of analytical methods is also influenced by the requirements of the diagnostic aim. The reference values of these tumour markers in human medicine determined in the Institute of Nuclear and Molecular Medicine

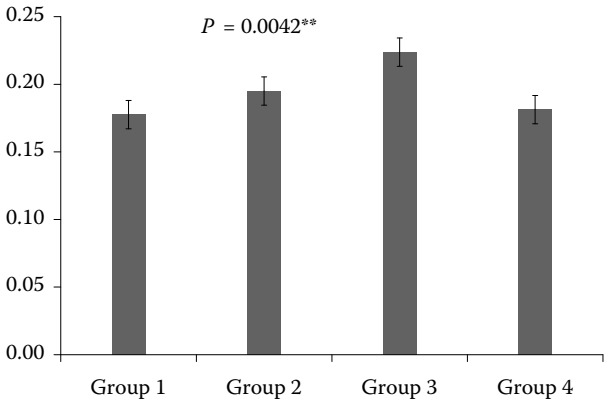

Figure 1. Values of CEA (ng/ml) markers in clinically healthy bitches (n = 32)

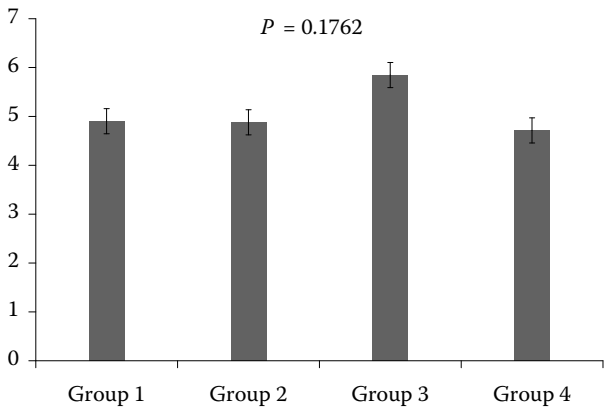

Figure 2. Values of CA 15-3 (IU/ml) markers in clinically healthy bitches (n = 32)

in Kosice were: CEA markers – up to 5 ng/ml, CA 15-3 markers normal values – up to 30.0 IU/ml, and cut-off values – 30.0–35.0 IU/ml. In the literature, values determined by other methods are as follows: 2.0 ng/ml for CEA markers and 25 IU/ml for CA 15-3 markers according to Ebeling et al. (2002). According to Laessig et al. (2007), the average values of CEA and CA 15-3 markers were 2.7 ng/ml and 43.8 IU/ml, respectively. In healthy subjects, the highest calculated values of CA 15-3 markers were 20.11 IU/l and 3.88 ng/ml for CEA markers (Park et al. 2008). These values are valid for human medicine.

In our study, we decided to categorise the dogs according to weight rather than breed. Also, we wanted to find a suitable modified method to determine these markers in healthy bitches by use of human kits. Determination of tumour markers in specific breeds or in specific age groups was not carried out because of the insufficient number of patients in our clinic. A more detailed comparison of results in these groups requires a substantially larger group of dogs where it is suitable to perform examinations of clinically ill animals. Thus, we consider our experimental values of CEA and CA 15-3 markers to be informative but preliminary. In our subject group, neither the weight nor age of the animals had an influence on the measured physiological values.

Our aim was to develop another simple diagnostic method for the diagnosis of mammary gland tumours in bitches before manifestations of clinical signs or postoperative diagnosis, and prior to total bilateral mastectomy. We pursued this avenue based on owners' questions regarding mammary gland tumours. It can be said that the measured values compared with human medicine are one tenth of the human value for CEA and approximately one fifth for the CA 15-3 markers. The results of our study could be the basis for the determination of CEA and CA 15-3 markers in bitches with mammary gland carcinomas. The study of these antigens in bitches with mammary gland carcinomas is ongoing.

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