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REVIEW

The canine alkaline phosphatases: A review of the isoenzymes in serum, analytical methods and their diagnostic application

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Abstract

This paper reviews the alkaline phosphatases in canine serum, the analytical methods used for qualitative and/or quantitative detection of these isoenzymes, and the diagnostic significancy of each of these isoenzymes. The paper further describes some of the latest advances in our knowledge of the canine alkaline phosphatases and possible areas of future research.

Key words: Canine, alkaline phosphatase isoenzymes, serum

Introduction

Alkaline phosphatase (ALP) (E.C. 3.1.3.1; orthophosphoric phosphohydrolase) consists of a group of heterogeneous isoenzymes that catalyze the hydrolysis of monophosphate esters at alkaline pH[21)]. The heterogeneity of these isoenzymes is partly due to the expression of different gene loci and partly to post-translational modifications[10,29)]. The ALP isoenzymes are glycoproteins and at least four gene loci are reported to encode their protein moieties in man (Table 1): the L/B/K locus determines the so called liver/bone/kidney or "tissue non-specific" ALP and is expressed virtually in all tissues; the intestinal locus is expressed in the intestinal mucosa localized to the brush borders of epithelial cells; the placental locus determines placental ALP characteristically expressed in syncytiotrophoblasts during gestation and in very small amounts in the lung and cervix but a similar isoenzyme variant called "Regan" rises in malignancies of lungs, gastrointestinal tract, ovary and uterus; and the placental like locus determines the so called "placental like" ALP which is similar but not identical in structure and properties to placental ALP and found in very small amounts in the testis and thymus of healthy individuals[7)] and a variant called "Nagao" in seminomas and a proportion of other turmors[10,13)].

In the dog separate ALP isoenzymes have also been isolated from the kidney, placenta, intestinal mucosa, liver and bone[27,31)]. Additionally, dogs have a unique isoenzyme secreted by the liver as a result of exposure to glucocorticoids called the corticosteroid-induced ALP (CALP) isoenzyme[2,3,35)]. Two genes are known to code for the canine ALPs (One for the ALP originating from the liver, kidney, bone and placenta; and another for the ALP from the

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Table 1. The encoding genes of the alkaline phosphatase isoenzymes and the exact tissue location in man

Gene	Isoenzymes	Tissue location
Tissue non-specific (TNS)	Liver*	
	Bone*	All tissues
	Kidney	
Intestinal	Intestine	Intestinal mucosa
	Corticosteroid induced*	Liver
Placenta	Placenta	Placenta, Lungs, Cervix
	Regan#	Lungs, GIT, Ovary, Uterus
Placenta-like	Placenta-like	Testis, Thymus
	NagaoΨ	Turmors

Variant of Placenta found in malignancies of lungs, GIT, ovary and uterus of humans.
Ψ Variant of Placenta-like found in seminomas and other turmors of humans.
* Found in canine serum.
GIT, Gastrointestinal tract.

intestine and includes the CALP isoenzyme). Characteristic differences observed in man in the expression of the various loci in different tissues and indeed in different cells within the same tissue have also been reported in dogs, but the reasons for these differences are still largely unknown[18].

Alkaline phosphatase has received the most study of all enzymes since the discovery of its clinical significance in the 1920s, but it is probably still the least understood of the commonly used serum enzymes[21]. Not only is the diagnostic value of the enzyme under constant study, but the physiological role of the enzyme has received a tremendous amount of interest. During the last few years, remarkable advances in our knowledge from our experiments have occurred[8,36,39,40,41]. In this paper, we review some advances in our knowledge and deal specifically with ALP isoenzymes in serum, analytical methods of ALP isoenzymes in serum and their diagnostic applications.

ALP isoenzymes in canine serum

In canine serum, three isoenzymes of ALP are known and these include liver ALP (LALP), bone ALP (BALP), and CALP[17]. Only two genes code for canine ALPs, namely: intestinal for intestinal ALP (IALP) and CALP; and the tissue non specific (TNS) gene for liver/bone/kidney isoenzymes, thus only two true isoenzymes exist[37]. The other ALP isoenzymes normally found in tissue namely, renal, placenta and intestinal isoenzymes have never been demonstrated in serum, neither in healthy nor diseased dogs. This has been attributed to their very short half-lives of about 3–6 minutes as opposed to approximately 3 or more days for LALP, BALP and CALP[19,35]. Their attachment to sialic acid was found responsible for the longer half-lives of LALP, BALP and CALP. It was further found to be responsible for their greater anodal migration on electrophoresis. Removal of sialic acid from LALP and CALP with neuramidase resulted in a dramatic reduction in the anodal migration and a reduction in the half-lives from approximately 3 days to less than 6 minutes[19].

The ALP isoenzyme composition in serum is known to be influenced by many factors. Age[29,44] and feeding status have particularly been noted to influence the isoenzyme composition as well as the total ALP activity in serum. BALP comprises approximately 96, 38, and 26% of TALP in young (under 1 yr.), middle aged (1yr.–7 yrs.) and old dogs (above 7 yrs.) whereas CALP is 12, 11, and 27% respectively. LALP increases steadily throughout life i.e, comprises less than 10% of TALP in young dogs but rises to more than 50% in middle aged and old dogs[40]. On the other hand, feeding and more particularly its resultant effect on carbohydrate levels has been noted to alter ALP levels in serum. Reducing sugars can react physiologically with amino acid groups including those of ALP in vivo and in vitro by a non-enzymatic glycosylation thus inhibiting the activity of these aminuteso acids[32]. It is therefore common to observe feeding related alterations in the ALP activity. Sex or breed-related differences in serum ALP are either small or do not occur at all in dogs[22] but higher levels

have been reported in Scottish terriers than other breeds[14]. Sex related differences, when they occur, are less important in the interpretation of results than are the effects of age.

Analytical methods

Due to the close similarities between the ALP isoenzymes several methods have been employed to distinguish between individual isoenzymes. Four general methods have proven particularly useful: thermostability studies; electrophoresis; differential inhibition with various aminoacids, small peptides and other low molecular weight substances; and immunologic methods (Table 2).

Table 2. A summary of analytical methods used to distinquish individual ALP isoenzymes

Thermostability	65°C×2min*
	56°C×10min
Electrophoresis	Cellulose acetate including affinity*
	Gel (Agar, Agarose, Starch, Polyacryldmide)
	Isoelectric focusing on agarose
Differential inhibition	Chemical (L-phenylalanine, L-homoarginine, L-phenylalanylglycylglycine, L-leucine, levamisole*)
	Glycoproteins (wheat germ lectin*)
Immunologic	Antisera raised against a specific isoenzyme individually or as part of electrophoresis

*Commonly used method(s) in the group

Thermostability: There is a marked difference in heat stability at 65°C for 2 minutes between isoenzymes coded by the intestinal gene and those by the TNS gene[26,43]. Of the 3 ALP isoenzymes in canine serum, CALP is the most resistant to heat inactivation followed by LALP and then BALP[6,7,26,30]. Heat inactivation can therefore, adequately differentiate CALP from BALP and LALP but differentiation of BALP from LALP although achievable is not reliable. The temperature coefficients for the inactivation of human LALP and BALP in serum by heat are 40 and 44, respectively[47]. Therefore, slight variations in temperature between measurements can have effects on rates of inactivation that are sufficiently great to obscure the small differences in stability between the two isoenzymes.

The heat inactivation method involves precise control of temperature by use of a large-volume, well-stirred water bath controlled by mercury-in-glass contact thermometer, and by introducing small volumes of the enzyme samples rapidly into small, thin walled glass tubes previously placed in the bath and allowed to reach temperature equilibrium. The tubes are promptly transferred to an ice bath at the end of incubation and the remaining activity in the samples determined.

Rates of inactivation of isoenzymes by heat can also be affected by changes in many other factors, notably pH, protein concentration, and the concentrations of substrates and cofactors[30]. For these reasons, routine measurement of ALP isoenzymes in serum and reproducibility of results using heat stabilities in a clinical laboratory is not reliable.

Electrophoresis: Zone electrophoresis followed by staining for enzymatic activity (usually with α-naphthyl phosphate as a substrate and with diazolium salt to detect the liberated α-naphthol) is the most useful single technique for the qualitative analysis of the multiple forms of ALP in serum. Various supporting media are used, each having its particular advantages[28]. Cellulose acetate electrophoresis has been recommended and widely used on canine sera[16,17]. However, conventional cellulose acetate electrophoresis separation may result in overlapping of ALP isoenzymes, making interpretation difficult[41,43]. Separation of the ALP isoenzymes can however, be improved by using cellulose acetate affinity electrophoresis[23,41]. By this method, the cellulose acetate membranes are presoaked in Tris-barbital buffer (pH 8.8) containing wheat germ lectin (WGL) at 50 mg/L for 20 minutes at room temperature. The isoenzymes are then separated by cellulose acetate electrophoresis at 180 V for 20 minutes. The

ALP bands are stained in buffer containing naphthol AS-MX phosphate sodium, fast blue RR salt and $MgSO_4$ and incubated for 25 minutes until bands are visible and proportioned in a densitometer. Lectin alters the isoenzyme migration pattern by binding to N-acetylglucosamine residues of the glycoproteins thus enhancing separation. The difference in the migration patterns of isoenzymes with lectin results from the heterogeneity in the surface carbohydrates of the various isoenzymes. Affinity electrophoresis is relatively simple, inexpensive, repeatable, easily interpretable and may be used routinely in a clinical laboratory, but it also remains largely qualitative especially when determining isoenzyme activity in normal canine serum samples. Although cellulose acetate is the most commonly used medium, the other methods using other support media such as agar gel, agarose gel, starch gel or polyacrylamide gel are available. Many of these gels yield greater separability of serum isoenzymes than does cellulose acetate, but in the clinical laboratory cellulose acetate remains the most useful method. Polyacrylamide electrophoresis has not been used extensively for ALP isoenzymes in animal studies.

Isoelectric focusing which increases resolution of ALP isoenzymes by causing proteins to migrate to their isoelectric points[4] on agarose has also been used but the method requires expensive equipment which may prohibit its routine usage in diagnostic laboratories.

The choice of a particular electrophoresis system is influenced by resources and skills available in a particular laboratory. But whatever system is chosen, consistent technique and experience in its use are necessary for reliable and routine identification of the various ALP zones. Furthermore the pH, buffer, media and voltage used should always be mentioned to enable comparisons of data to be easily done.

Differential inhibition: Experiments on human serum have shown that various low molecular weight substances can differentially inhibit ALP isoenzymes. Five inhibitors have been extensively used and these include: L-phenylalanine which inhibits the intestinal isoenzyme including CALP, placental and placental-like isoenzymes; L-homoarginine that inhibits liver/bone/kidney isoenzymes; L-phenylalanylglycylglycine which inhibits the intestinal isoenzyme including CALP, placental and placental-like isoenzymes; L-leucine which inhibits the intestinal including CALP, placental and placental-like isoenzymes; and levamisole or its analog p-bromotetramisole which inhibits liver/bone/kidney isoenzymes[13,20,24,41]. Most of these inhibitors have been used in quantitative procedures but none except for levamisole at 4.2 mM has been automated and gained widespread use in most diagnostic laboratories including ours[20,41]. Levamisole is rather cheap and easy to adapt for use in most autoanalyzers and can be used to accurately and routinely quantify the CALP isoenzyme activity as part of the biochemical profile.

Other substances widely used to differentially quantify ALP isoenzymes in serum are lectins[1,6,7,8,34]. Lectins are proteins or glycoproteins that precipitate carbohydrates because of their highly specific carbohydrate binding properties. Alkaline phosphatases are also glycoproteins with varying carbohydrate chains to which the lectins can bind. Of the lectins, WGL has been extensively used to quantify BALP. WGL recognizes sialic acid and N-acetylglucosamine of human BALP[1,34], equine BALP[12] and canine BALP[41] and selectively precipitates this isoenzyme from serum. The precipitated portion can then be accounted for by determining the remaining activity and subtracting it from the total activity before the lectin was added. Combining WGL precipitation and levamisole inhibition as part of one assay has been used in a semiautomated procedure for quantifying ALP isoenzymes in canine serum for routine

use in a diagnostic laboratory[41].

Immunologic methods: Immunologic methods have not found widespread use in canine ALP isoenzymes as they have on human ALPs. Antisera have been raised against CALP and found to differentiate the isoenzyme from LALP. This antisera's cross reaction with the intestinal ALP led to the conclusion that the CALP and intestinal ALP are antigenically similar to each other, but different from LALP[46]. In humans, antisera raised in rabbits against one ALP isoenzyme have been found to cross react with the others[25]. Extensive studies with monoclonal antibodies raised against each of the different ALP isoenzymes, have provided a wealthy of information about the antigenic determinants present on each of the different molecular forms. Combinations of these various biochemical and immunological techniques have been used to devise methods which give analytical information about the quantities of each of the ALP isoenzymes when they are present together in a tissue or body fluid such as serum. In canine serum for instance, CALP has been quantified using sepharose beads to which antibody directed against CALP has been attached. AntiCALP sera has also been employed as part of the electrophoresis procedure to enhance seperation of isoenzyme bands that normally wouldn't separate under conventional cellulose acetate electrophoresis[45,46].

Immunologic methods are costly because of the limited commercial availability of specific ALP isoenzymes and respective antibodies. Furthermore, the methods have not been automated thus they can not be used routinely in a clinical laboratory.

Diagnostic application

The diagnostic role of the ALP isoenzymes in canine serum is summarized in table 3.

Bone ALP: BALP is present in osteoblasts as an ectoenzyme and has been shown to play a role in bone formation but its precise biochemical function is unknown. Previous studies indicate that quantification of BALP can provide an index of the rate of bone formation[1,5,7,9,37,38]. Growing animals have been observed to have high BALP activity resulting from active skeletal development[41,44]. Increased BALP has also been observed in osteosarcoma[17,33,41,48,49]. However, BALP does not always increase in tumors involving the bone and when it does maximum elevations of only 2 to 3 times normal may occur. This is basically because it is associated with osteoblastic activity which is not a common feature of all bone tumors. Even osteosarcomas are of different biochemical behavior i,e. osteoblastic, chondroblastic etc. and thus BALP will only increase in the osteoblastic type[49]. Measuring BALP has proved useful in differentiating osteoblastic from other types of osteosarcoma[41]. Raised BALP activity has also been observed in naturally occurring nutritional osteopathies such as rickets and osteomalacia[11,15] and in secondary renal hyperparathyroidism. Fractures have also been implicated but our studies[41] have shown no indication of increased BALP in dogs with fractures.

Liver ALP: The isoenzyme has been associated with liver disease and most frequently as a marker of cholestasis. The isoenzyme activity

Table 3. The diagnostic role of the ALP isoenzymes in canine serum

Isoenzyme	Condition
Bone	Bone formation*
	Osteosarcoma*
	Nutritional osteopathies (rickets, osteomalacia)?
	Secondary hyperparathyroidism
	Fractures?
Liver	Liver disease (cholestasis*, fatty liver,
	malignant tumors, hepatoma, cirrhosis)
	Others (copper toxicity, portosystemic vascular
	shunt etc.)?
Corticosteroid-induced	Steroid hepatopathy
	(iatrogenically or endogenously)

? Insufficient evidence

*Most common cause

increases in cells lining the bile canaliculi and sinusoids as a result of direct induction and the hepatotoxic effect of bile acid that drastically increase during cholestasis.

Other liver conditions that have been observed to result in elevated LALP include steroid hepatopathy, fatty liver, malignant liver tumors both primary and secondary, hepatoma, and cirrhosis[2]. In liver disease, the concurrent estimation of alanine aminotransferase (ALT) improves the differential diagnosis between conditions leading to acute hepatocellular damage in which ALT increases but LALP does not and those that do not[2,21]. Raised LALP has also been reported in copper toxicity, bile duct rupture, suppurative cholangiohepatitis, and portosystemic vascular shunts[2,14,21,22,27,31].

Clinically, LALP is not definitive for any one particular disease but combined with a concurrent examination of other liver enzymes, the LALP activity in serum has proved to be a useful parameter in differential diagnosis of liver diseases.

Corticosteroid-induced ALP : CALP is produced in the liver in that area of the hepatocyte that comprises the bile canaliculi and solubilized in serum by an enzyme, phospholipase[42]. CALP increases in dogs treated with glucocorticoids, in those with spontaneous or iatrogenic hyperadrenocorticism, in dogs with hepatic or nonhepatic neoplasia, and, most important, in dogs with many different chronic illnesses[3,23,41]. Increased activity in turmors is more a result of the turmor's induction of stress and the resultant effect on the increased endogenous glucocorticoid secretion than it being a turmor marker. Several studies have evaluated the usefulness of CALP in diagnosing hyperadrenocorticism and have come up with a rather uniform agreement[2,3,16,23]. That CALP is increased in most dogs with Cushing's and as such, the test is considered sensitive. Finding increased CALP is however, nonspecific and may be a result of one of the three disorders commonly confused with naturally occurring hyperadrenocorticism : iatrogenic Cushing's, diabetes mellitus and hepatopathies[2,3,16,23]. It is usually concluded that finding low CALP levels is a definitive diagnosis for ruling out hyperadrenocorticism. It has also been observed that CALP may be used to screen for the severity of steroid induced vacuolar hepatopathy. The degree of steroid-induced hepatopathy was quantified by vacuolization scores and digital histogram analysis of B-mode ultrasonographs[39,40]. Results indicated that regardless of how high total serum ALP is, if the activity is predominantly due to CALP the level of hepatopathy is more severe than that when LALP is predominant. This was due mainly to the fact that, apart from the bile canalicular sites, only severely vacuolized cells were found to be predominantly involved in CALP secretion. The isoenzyme should therefore, be tested further for its potential to screen severity of hepatopathy during long term glucocorticoid therapy.

Conclusion.

It is remarkable that despite extensive studies of the various ALP isoenzymes over more than four decades, so little is known, with any certainty, about their biological functions in the normal organism. Canine serum ALPs are of limited value compared to their counterparts in humans despite being the commonly assayed serum enzymes in the clinical laboratory. This has largely been due to lack of information on the presence of variants commonly observed in humans that are associated with cancer. Future studies may have to concentrate on finding such definitive variants through improvement of assaying techniques or analyzing similar cases as those in humans where the variants are detectable.

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