EVALUATION OF RECOMBINANT LIG ANTIGEN-BASED ELISA FOR DETECTION OF LEPTOSPIRAL ANTIBODIES IN CANINE SERA

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Abstract. The objectives of this study were to clone the conserved region of leptospiral immunoglobulin-like protein (*lig*) gene and evaluate the utility of the recombinant Lig as an ELISA antigen for detection of leptospiral antibodies in canine sera. *Leptospira kirschneri* serovar Grippotyposa strain Moskva V was chosen to be a target for cloning the conserved region of Lig gene. This assay was evaluated with canine sera (n = 91) that were MAT-negative (<1:100 dilution) and sera (n = 103) that were MAT-positive (≥ 1:100 dilution) using 24 serovars. The ELISA showed a relative sensitivity as compared to MAT of 84.5% whereas the specificity was 76.9%. This assay is simple and can be routinely prepared in large amounts. It was concluded that the GST.Lig recombinant protein-based ELISA could be used as a screening test for serodiagnosis of canine leptospirosis with also for confirmation of MAT-positive test results.

Keywords: leptospirosis, recombinant Lig, ELISA, canine

INTRODUCTION

Leptospirosis, a zoonosis caused by *Leptospira* bacterium, occurs worldwide and can cause mild to severe health problems in both humans and animals, particularly in areas with hot and humid climate (Levett, 2001; Bharti *et al*, 2003). Pathogenic leptospires, living in the kidneys of a wide range of animal hosts, most notably rodents, insectivores, and cattle, can be transmitted most commonly through urine of those hosts (Bharti *et al*, 2003). Because the disease involves several types of leptospires and several kinds of animal hosts, control of the disease is complicated. In addition, because leptospiral infection in animals is often associated with non-specific symptoms, definitive diagnosis of the disease must be based on both symptoms of infected animals and results of serological tests.

Microscopic agglutination test (MAT)

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is the most common serological test used for laboratory diagnosis of letospirosis. However, MAT provides some disadvantages: requiring live antigens which may be restricted to some laboratories; yielding false-negative results especially in cases of antibody titers induced by vaccination and in cases of early stage of the disease; and giving cross-reactivity (Fain *et al*, 1999; Pamela, 2008). On the other hand, enzyme-linked immunosorbent assay (ELISA), provides some advantages: more specificity, thereby reducing false-positives associated with vaccine response, and the ability of handling many samples at one time (Ribotta *et al*, 2002; Surujballi *et al*, 2004).

Information on the nature and identity of leptospiral antigens is important for elucidation of their significance in the immunity of leptospirosis and for pathogenesis and diagnosis of leptospirosis. Palaniappan *et al* (2002) were the first to identify and characterize immunogenic *Leptospira* proteins that are expressed during infection. The gene for one such immunoreactive immunoglobulin-like 130-kDa protein (LigA) of *L. interrogans* serovars Pomona type Kennewicki has been characterized and shown to be expressed *in vivo* (Palaniappan *et al*, 2002). Lig may play a role in host cell attachment and invasion during leptospiral pathogenesis and for the strong antibody response in patients and infected animals (Palaniappan *et al*, 2002, 2004; Matsunaga *et al*, 2003; Koizumi and Watanabe, 2004). It can be used to differentiate between vaccinated and naturally infected animals (Matsunaga *et al*, 2003). Therefore, identification of Lig that is expressed only *in vivo* may provide new insights for developing strategies to improve diagnosis, vaccination and treatment protocols (Palaniappan *et al*, 2002, 2004; Croda *et al*, 2007; Srimanote *et al*, 2008).

The purposes of the this study were to clone and express the conserved region of Lig from *Leptospira kirschneri* serovar Grippotyphosa strains Moskva V as GST-fusion protein in *Escherichia coli* and to develop and evaluate the utility of the recombinant Lig protein as antigen in an indirect ELISA for the detection of antibodies against *Leptospira* in canine sera.

MATERIALS AND METHODS

Production of Lig as a coating antigen

Source of microorganism. Twenty-three *Leptospira* serovars from 7 pathogenic species (*L. interrogans, L. kirschneri, L. meyeri, L. borgpetersenii, L. weilii, L. noguchi, L. santarosai)*, a non-pathogenic species (*L. biflexa)* were obtain from Department of Veterinary Public Health and Diagnostic Health, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand (Table 1) and were grown in Ellinghausen and McCullough medium as modified by Johnson and Harris in continuous culture (Fain *et al*, 1999).

Cloning of Lig gene. The gene encoding conserved region (LigA and LigB) was constructed to contain the N-terminal 576 amino acids without signal sequence. The gene was amplified from genomic DNA of *Leptospira kirschneri* serovar Grippotyphosa strains Moskva V using primers designed with restriction enzyme sites for subsequent cloning of *lig* based on the *L. kirschneri* Lig sequence (GeneBank accession number AY 190126), namely forward primer 5′ CGTGTCGACGTGAAGAAAA TATTTTGT ATTTCGATTTT CC 3′ and reverse primer 5′ CCTCGAGGGGATAA CGTAGAAA CCGGACTAC 3′. Forward

and reverse primer included a *Sal*I site and *Xho*I site, respectively (underlined). The purified 1,728 bp fragment of *lig* was inserted into *Sal*I-*Xho*I sites of expression vector pGEX-5X-3 (Amersham Pharmacia Biotech, Piscataway, NJ) producing a Lig fusion protein with glutathione-S-transferese (GST) and transfected into *E. coli* BL21. To confirm that the correct sequence of the insert, direct sequence analysis was performed using BigDye Terminator Cycle Sequence Ready Reaction kit (Applied Biosystems, Carlsbad, CA).

As the GST fusion protein was expressed as inclusion bodies; the method for preparative purification of insoluble GST fusion protein was then performed. The expressed protein was pelleted, washed once with ice-cold STE (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA), resuspended by repeated pipetting in STE containing 100 µg/ml of lysozyme and incubated on ice for 15 minutes. Bacteria were lysed by addition with various urea concentration (1 to 8 M), cells were sonicated on ice and recovered by centrifugation. The supernatant was transferred to a new tube, sarkosyl was added to the desired final concentration (0.1-2%) from a 10% stock in STE. The purity of GST fusion proteins was assessed by SDS-gel-electrophoresis and staining with Coomassie brilliant blue. Protein concentration was determined using Bradford method (Bradford, 1976). The rLig and pGEX-5X-3 expression proteins were used as antigens for detection of anti-leptospiral antibodies.

Antigenicity of Lig. Purified GST-Lig fusion (GST.Lig) and GST proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes which then were incubated in blocking solution [5% skim milk in phosphate-buffer saline (PBS) supplemented with 0.05% Tween 20 (PBS-T)] at 37ºC for 1 hour. Canine sera (primary antibodies from MAT-positive and MAT-negative sera) were pre-adsorbed with lysate from transformed *E. coli* BL21 containing pGEX-5X-3 for 1 hour at 37ºC, before incubating (diluted 1:40) with membranes at 37ºC for 1 hour. Membranes were washed 3 times with PBS-T and then incubated with Protein A-horseradish peroxidase (HRP) conjugate (ZYMED, South San Fransisco, CA) (diluted 1:1,000 (v/v) in 1% skim milk in PBS-T) at 37ºC for 30 minutes, and washed 3 times in PBS-T. Membranes were developed with 3,3′-diaminobenzidine tetrahydrochloride.

Test sera

Source of samples. A total of 194 canine serum samples were provided from Leptospirosis Research Laboratory, Department of Veterinary Public Health and Diagnostic Service, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand. Ninety-one sera with MAT negative titers at a dilution of 1:100 were used to determine the relative specificity of ELISA. For determination of relative sensitivity of ELISA, 103 canine sera showing MAT titers ≥ 100 positive to leptospiral serovars were employed.

Classification of sero-status by MAT. MAT was performed as previously described by Cole *et al* (1973) with all 24 serovars of *Leptospira* spp used as antigens. Titer is the reciprocal of the highest dilution of serum with 50% agglutination or lysis of leptospires.

ELISA protocol

Purified GST. Lig and GST (negative control) proteins were diluted in a coating buffer (0.007 M Na_2CO_3, 0.017 M $NaHCO_3$, pH 9.6) at optimum concentration established by checkerboard titration. One

hundred microliters of the diluted antigen were added into each well of a 96-well microtiter plate (MaxiSorb ImmunoPlates; Nunc, Rochester, NY), and the plate was incubated at 4ºC overnight and then washed 3 times with PBS-T. Plates were incubated in blocking solution (5% dry skim milk in PBS-T) at 37ºC for 1 hour. Canine sera (primary antibodies from MAT positive and negative sera) were adsorbed as previously described. Then the wells were incubated with 100 µl of canine sera (diluted 1:40) at 37ºC for 1 hour. After 3 washes with PBS-T, wells were then incubated with 100 µl of Protein A-horseradish peroxidase conjugate (ZYMED, South San Franscisco, CA) diluted to 1:10,000 (v/v) in 1% skim milk in PBS-T at 37ºC for 30 minutes. Following 3 washes with PBS-T, wells were incubated with TMB substrate (ZYMED, South San Francisco, CA) for 10 minutes at room temperature. Absorbance at 650 nm was measured using an ELISA reader (Rosys Anthos Lucy 2).

Statistical analysis

Determination of cut-off value. An average absorbance value at 650 nm (A_{650}) ≥0.177 is considered positive.

Test performance. The relative sensitivity, specificity and accuracy of ELISA for the detection of anti-leptospiral antibodies in dog sera were determined in comparison to the MAT as follows. Sensitivity = a/(a+b) x 100; Specificity = d/ (c+d) x 100; Accuracy = [(a + d)/(a + b + c + d)] x 100.

Where a is the number of samples positive by both ELISA and MAT; b is the number of samples positive by MAT but negative by ELISA; c is the number of samples negative by MAT but positive by ELISA; and d is the number of samples negative by both MAT and ELISA.

RESULTS

Production and purification of recombinant Lig antigen

The designed PCR primers were able to amplify 1,728 bp *lig* of *Leptospira kirschneri* serovar Grippotyphosa strains Moskva V (Fig 1A). The DNA and amino acid sequence showed 92-96%, 93-96% similarity with *lig* in GenBank (GenBank numbers AY190126, AY098156 and AY098157). The nucleotide sequence of *lig* from *Leptospira kirschneri* serovar Grippotyphosa strains Moskva V has been assigned GenBank accession number EF 517920.

Expression of *lig* in *E.coli* after growth and inducted with 0.1 mM isopropyl-β-D thiogalactopyronoside (IPTG) was demonstrated by the presence of Lig protein of 96 kDa (26 kDa for GST protein plus 70 kDa for Lig protein) in SDS-PAGE (Fig 1B). The expressed protein was present in the insoluble fraction (Fig 1C). Of all the conditions examined for inclusion fraction solubilization, the GST fusion protein can be solubilized by 4 M urea and 1% sarkosyl (Fig 1D).

Detection of anti-Lig antibodies in canine serum

In order to confirm the antigenicity of the recombinant Lig protein, purified GST.Lig and GST at the same molar ratio were separated on 12% SDS-PAGE, and immunoblot assay was performed using canine sera. Fig 2 shows a representative result of the immunoblot analysis: 3 MAT-positive canine sera reacted with the GST.Lig fusion protein but not with GST and 3 MAT-negative serum did not react with both GST.Lig and GST proteins.

Optimization of recombinant antigen concentration

A checkerboard titration technique

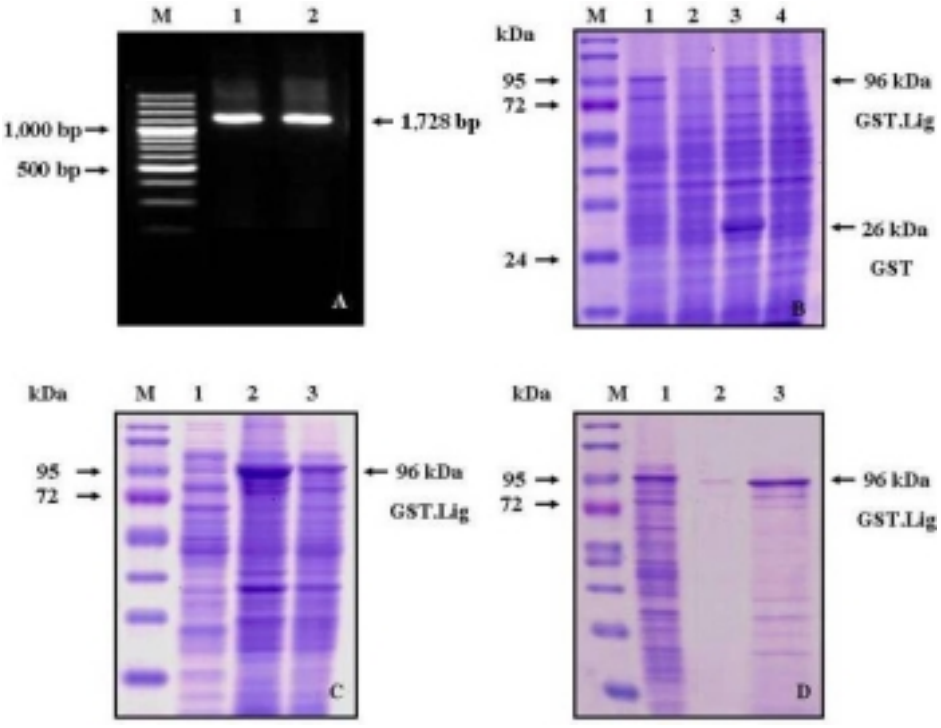

Fig 1–Gel electrophoresis of PCR amplification of Lig gene and heterologous express recombinant Lig protein. A. Agarose gel. Lane M, standard marker (500 bp); lanes1-2, 1,728 bp of Lig gene. B. SDS-PAGE analysis of recombinant Lig protein. Lane M, molecular weight standards; lane1, expression of recombinant Lig protein (96 kDa); lane 2, expression of negative control cell; lane 3, expression recombinant GST (26 kDa); lane 4, expression of negative control cells. C. SDS-PAGE analysis of characterization of fusion protein. Lane M, protein molecular weight markers; lane 1, supernatant of GST.Lig-expressing cells; lane 2, pellet of GST.Lig- expressing cell; lane 3, IPTG induced cells expressing GST.Lig. D. SDS-PAGE analysis of protein solubilization of GST.Lig. Lane M, protein molecular weight markers, lane 1, pellet of GST.Lig-expressing cells; lane 2, supernatant of GST.Lig-expressing cells, lane 3, insoluble pellet of GST.Lig expressing cell after solubilization with 4 M urea and 1% sarkosyl.

was used to determine the optimum concentrations of reagents for ELISA. Using the strongest MAT-positive canine serum, the optimum concentration of recombinant antigen yielding high specificity was obtained at 5 µg and the optimum dilution of primary and secondary conjugated antibodies was assessed at 1:40 and 1:10,000, respectively. As the recombinant antigen was expressed as GST-fusion protein, GST was used as negative control and the reactivity of GST was deducted for analysis of samples.

MAT

A titer equal to or higher than 1:100 against one or different serovars was considered positive. In most of seropositive cases, multiple titers against different serovars were detected. In seropositive dogs the predominated titers against pathogenic *Leptospira* were serovars

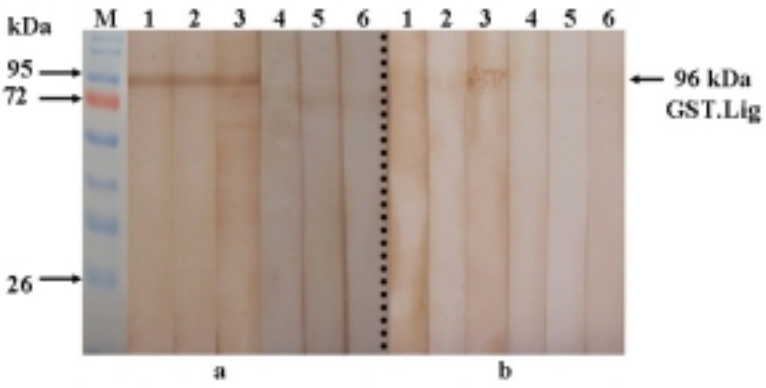

Fig 2–Anti-recombinant Lig antibodies in sera from canine leptospirosis. Membranes were prepared from SDS-PAGE of recombinant protein fragment of *L. kirschneri* serovar Grippotyphosa strain Moskva V Lig conserved region of 96 kDa (a) and GST protein of 26 kDa as control (b). Membranes were incubated with 1:40 dilution of MAT-positive (lanes 1 to 3) and MAT-negative canine sera (lanes 4 to 6) followed by 1:1,000 dilution of HRP-conjugated protein A. Antibody binding was visualized by exposure to DAB® peroxidase substrate. M, standard protein marker.

Tarassovi, Icterohaemorrhagiae, Sarmin, Canicola, Ranarum, Bataviae, Shermani, Sejroe and a non-pathogenic strain (Patoc I). Other serovars were almost negative (data not shown).

Correlation between MAT serovar and ELISA result

Table 2 shows the frequency distribution of absorbance values of the rLig ELISA for 194 canine sera. The cutoff value of an indirect ELISA using rLig antigen was determined from 91 MAT-negative canine sera. The mean OD_{650} value was 0.065 with standard deviation (SD) of 0.028. The cutoff value for estimation of positive and negative samples was 0.177 (mean plus 4 SD). Sensitivity, specificity and accuracy of ELISA compared to MAT was 84.5, 77 and 81%, respectively (Table 3).

DISCUSSION

In the present study, recombinant antigen-based ELISA was developed and evaluated for the serodiagnosis of canine leptospirosis. The conserved region of recombinant Lig (rLig) protein was used for antigenic preparation. The ELISA developed in this study had a sensitivity of 84.5% relative to MAT when canine serum samples with MAT of ≥ 100 to difference serovars were tested.

Of the 91 MAT-negative sera tested, 21 samples were ELISA-positive. The specificity of ELISA (77%) may be an underestimation of the true specificity of the indirect ELISA. MAT-negative sera may contain non-agglutination leptospiral antibodies, which were detected by ELISA but not by MAT as MAT can detect only agglutinating antibodies (Ribotta *et al*, 2002).

Some MAT-positive canine serum samples were found to be negative by ELISA. Several studies have demonstrated that the agglutinins produced are mainly IgM (Adler *et al*, 1980; Surujballi *et al*, 1997), and other studies have reported very few

Table 1
Reference strains of *Leptospira* used.

Species	Serogroup	Serovars	Strain
L.interrogans	Autumnalis	Autumalis	Akiyami A
	Batavia	Bataviae	Swart
	Australis	Bratislava	Jez Bratislava
	Canicola	Canicola	Hond Utrecht IV
	Djasiman	Djasiman	Djasiman
	Hebdomadis	Hebdomadis	Hebdomadis
	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
	Pomona	Pomona	Pomona
	Pyrogenes	Pyrogenes	Salinem
L. noguchii	Louisiana	Louisiana	LSU 1945
	Panama	Panama	CZ 214
L. borgpetersenii	Ballum	Ballum	MUS 127
	Javanica	Javanica	V. Batavia 46
	Mini	Mini	Sari
	Sejroe	Sejroe	M 84
	Tarassovi	Tarassovi	Perepelitsin
L. santarosai	Shermani	Shermani	1342 K
L. weilii	Celledoni	Celledoni	Celledoni
	Manhao	Manhao	Li 130
	Sarmin	Sarmin	Sarmin
L. kirschneri	Cynopteri	Cynopteri	3522 C
	Grippotyphosa	Grippotyphosa	Moskva V
L. meyeri	Ranarum	Ranarum	ICF
L. biflexa	Samaranga	Patoc	Patoc I

Table 2
Frequency distribution of optical density (OD_{650}) values indirect ELISA using recombinant Lig antigen of positive canine sera in comparision with MAT[a].

OD_{650}	No. of sera	
	MAT positive	MAT negative
0-0.038	10	33
0.039-0.084	2	11
0.085-0.130	2	11
0.131-0.176	2	15
≥ 0.177	87	21
Total (*n*)	103	91[b]
Mean OD ± SD		0.065 ± 0.028

[a]77 samples of MAT negative were used for calculation of mean OD_{650} values, and 14 samples with MAT-positive ($OD_{650} \geq 0.48$) were excluded; [b]The cut-off value for the interpretation of positive and negative samples was determined as the mean plus 4 SD (0.177), of the MAT-negative absorbance.

Table 3
Comparison between MAT and ELISA.

		MAT	
		Positive	Negative
ELISA	Positive	87	21
	Negative	16	70
	Total	103	91

MAT was considered positive at 1:100 dilution of serum. ELISA was considered positive at 1:40 dilution of serum when the absorbance was above the cut-off value (0.177).

animals and humans with leptospirosis produce IgG agglutinins that can be examined by MAT. However, the presence of IgG antibodies are detectable by ELISA. Presumably, they are directed against nonagglutinating antigens (Morris *et al*, 1974). Croda *et al* (2007) performed Western blot analysis to detected IgM and IgG antibodies using the recombinant N-terminal Lig as antigen and found that a high proportion of leptospirosis patients were found to have anti-Lig IgG antibodies during the acute phase of the illness. Srimanote *et al* (2008) used the C-terminal portion of recombinant LigA (cLigA) as reagent in an indirect ELISA for detecting IgM and IgG in sera of leptospirosis patients, with a diagnosis sensitivity of 100% for both cLigA IgM and IgG and specificity of 98% and 100%, respectively.

In this study, the results using the conserved region of recombinant protein Lig for the detection of anti-leptospirosis in canine sera conformed with previous reports. Palaniappan *et al* (2004) evaluated the diagnostic potential of conserved and variable regions of LigA and LigB (Con, VarA and Var) in a kinetic ELISA using MAT-positive canine sera. The conserved regions of LigA and LigB (rCon) show strong reactivity to MAT-positive canine demonstrating that this region appears to be specific for serodiagnosis of leptospiral infection and this antigen can be used to identify leptospiral infection despite vaccination. The lig protein is unique to pathogenic *Leptospira* spp and that *Leptospira*-infected host produce antibodies to Lig (Palaniappan *et al*, 2002; Matsunaga *et al*, 2003).

Commercially available vaccines containing Canicola and Icterohaemorrhagiae serovars are broadly used. In general, vaccinated animals develop relatively low agglutinating antibody titers (100-400) to those serovars in the vaccine and these titers persist for 1-3 months after vaccination (Carole and Bolin, 2003). However, some animals develop high titers after vaccination (particularly those vaccinated several time each year) and although these high vaccination titers decrease with time, they may persist for 6 months or more after vaccination. Introduction of new vaccines may also change the typical pattern of post-vaccination antibody titers (Bolin *et al*, 2003). Taken together, the lack of antibodies to recombinant Lig protein in these 21 MAT-positive serum samples suggests that MAT titers are due either to non-specific reactivity or vaccination. Eight samples were positive with antibodies titer ranging from 50 to 800 to serovar Canicola and/or Icterohaemorrhagiae (data not shown). It also must be considered that the antisera used in this study are defined as positive or negative for particular *Leptospira* serovar on the basis of MAT results alone. Since the vaccination status of these dogs was unknown, all titers against serovars Icterohaemorrhagiae and Canicola were considered positive in MAT-positive

group. Due to the difficulty and expense associated with the culturing of *Leptospira*, the results obtaining from culture samples were not available. Further studies using sera from dogs with both leptospiral vaccination and natural infection are needed to answer this question. Diagnosis of leptospirosis in animals is dependent on a good clinical and vaccination history. Coordination between diagnostic laboratory and veterinarian is required to make an accurate diagnosis.

In summary, the development and evaluation described in this study of recombinant antigen-based ELISA for the detection of canine anti-leptospiral antibodies resulted in a simple, rapid, sensitive and specific test for screening canine sera. The assay used a recombinant immunoglobulin-like protein of leptospira antigen whose expression is highly associated with pathogenic leptospiral infection. It is safer than MAT since it eliminates the handling of live leptospires. Thus, it is suitable for large scale serological examinations, routine diagnostics, epidemiology surveys and follow-up investigations of outbreaks. As the diagnosis of leptospirosis is based on a single serum sample, the ELISA described here would be particularly useful in canines with full clinical picture and vaccination history. However, studies are needed to assess the advantages and limitations of the test and the types of appropriate samples.

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