Abanico Veterinario. January-December 2021; 11:1-13. http://dx.doi.org/10.21929/abavet2021.31
Short Communication. Received: 12/11/2020. Accepted: 08/08/2021. Published: 28/08/2021. Code: e2020-90.

Development and validation of two immunoassays for the detection of *Brucella canis* in dogs

Desarrollo y validación de dos inmunoensayos para la detección de *Brucella canis* en perros

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ABSTRACT

Canine brucellosis is a cause of reproductive failure and early detection in infected dogs remains a challenge. The aim of the present study was to test two antigens to be used in two different immunoassays for the detection of Brucella canis infection in dogs: one from Brucella canis RM6/66 sonicated crude antigen (CA-iELISA) and the other using the immunodominant protein GroEL (GroEL-iELISA). The cut-off point was determined using sera from infected dogs; subsequently, reproducibility was measured and a coefficient of variation (CV) below 15 % was obtained for negatives and positives with the CA-iELISA, whereas, for the GroEL-iELISA they were higher than 15 %. In the robustness test, there were significant differences for the GroEL-iELISA, but not for the CA-iELISA. The selectivity test showed cross-reactivity with Leptospira interrogans in the CA-iELISA, and with L. interrogans and Salmonella spp. in the GroEL-iELISA. From the sample stability tests, it was demonstrated that samples should be stored at room temperature for no more than 2 hours, at 4 ºC for no more than 24 hours and can be kept at -20 ºC for up to 30 days. Finally, sensitivity and specificity were calculated, resulting in 100 % for both CA-iELISA; sensitivity of 44 % and specificity of 67 % for GroEL-iELISA. In conclusion, the CA-iELSA proved to be better at detecting Brucella canis than the GroEL-iELISA.

Keywords: *Brucella canis,* Canine Brucellosis, GroEL, ELISA.

RESUMEN

La brucelosis canina es causa de falla reproductiva y la detección temprana en los perros infectados sigue siendo un desafío. El objetivo del presente estudio fue probar dos antígenos para ser utilizados en dos diferentes inmunoensayos, para la detección de infección por *Brucella canis* en perros: uno a partir de antígeno crudo sonicado de *Brucella canis* RM6/66 (CA-iELISA) y otro utilizando la proteína inmunodominante GroEL (GroEL-iELISA). Se determinó el punto de corte usando sueros de perros infectados; posteriormente, se midió la reproducibilidad y se obtuvo un coeficiente de variación (CV) debajo de 15 % para negativos y positivos con el CA-iELISA, mientras que, para el GroEL-iELISA fueron superiores a 15 %. En la prueba de robustez, hubo diferencias significativas para el GroEL-iELISA, más no para el CA-iELISA. La prueba de selectividad mostró reacción cruzada con *Leptospira interrogans* en el CA-iELISA, y con *L. interrogans* y *Salmonella* spp. en el GroEL-iELISA. A partir de las pruebas de la estabilidad de la muestra se demostró que éstas deben almacenarse a temperatura ambiente no más de 2 horas, a 4 ºC no más de 24 horas y pueden mantenerse a -20 ºC hasta 30 días. Finalmente, se calcularon la sensibilidad y

especificidad, resultando 100 % ambas para el CA-iELISA; una sensibilidad de 44 % y especificidad de 67 % para el GroEL-iELISA. En conclusión, el CA-iELSA demostró ser mejor en detectar a *Brucella canis* que el GroEL-iELISA.
Palabras clave: *Brucella canis,* Brucelosis canina, GroEL, ELISA.

INTRODUCTION

Brucellosis is the most widespread bacterial zoonosis worldwide (Corbel, 2006). Nevertheless, it is still one of the diseases given the least consideration as a potential cause of chronic – degenerative conditions (Hull and Schumaker, 2018). Canine Brucellosis was described by Leeland Carmichael in 1966, after isolating the agent *Brucella canis* from Beagles with reproductive problems (Cosford, 2018). It is the most common cause of reproductive failure in dogs, (Hensel *et al*., 2018) mainly affecting household and shelter dogs (Hubbard *et al*., 2018) in which fever is accompanied by epididymitis, prostatitis, and reproductive failure, in addition to nonreproductive manifestations such as lymphadenitis, uveitis, endophthalmitis, pyogranulomatous dermatitis, meningoencephalitis, and discospondylitis (Cosford, 2018; Wanke, 2004). Detection is currently based on the isolation of the agent by bacterial culture and seroagglutination (Cosford, 2018). However, serological diagnosis is problematic in the identification of chronic cases, and culture presents a risk to the laboratory personnel (Hull and Schumaker, 2018). Therefore, there is a need to develop new methods for the detection of this microorganism, and serological methods are ideal given their ease of use and demonstrated reliability (Cosford, 2018; Hull and Schumaker, 2018). However, these methods require good quality control, and must therefore be validated before they are used in the field (Andreasson *et al*., 2015).

The aim of this study is to establish a serological method (I – ELISA) for the detection of *Brucella canis* antibodies in dogs, using as a capture antigen the 60 kDa chaperone protein GroEL, and a crude sonicated antigen (CA). Finally, to compare the performance of both immunoassays and to determine the conditions for its maximum utility.

MATERIAL AND METHODS

Animals and sera

For this study, we used sera from 10 beagle dogs experimentally infected with *B. canis,* obtained in a previous work, kindly donated by Efrén Díaz (CENID SAI; INIFAP SAGARPA, México) (Tuxpan Galván, 2015), confirmed by rapid slide agglutination test (RSAT) and bacterial isolation from blood samples. Briefly, animals were inoculated by instillation in the conjunctiva with 5 x 10^6 CFU/ml of a *B. canis* field strain, blood samples were taken at different days post infection and cultured in Ruiz-Castañeda medium. *Brucella canis* identification was performed by routine biochemical tests according to the methodology described by Alton (1988).

Sera from 10 clinically healthy dogs were used, whose last vaccination against any agent was at least six months prior to sampling and which gave a negative result in the RSAT (Carmichael and Joubert, 1987). For selectivity tests, we used three sera from dogs with *Leptospira interrogans* infections confirmed by the microscopic agglutination test, two sera from dogs recently vaccinated against *L. interrogans*, Canine Adenovirus type 1 and 2, and Canine Distemper, and serum from a positive dog for gastrointestinal infection with *Salmonella* spp., as confirmed by culture. All these sera came from the sera stock of the Microbiology and Immunology Department, Faculty of Veterinary Medicine at the National Autonomous University of Mexico.

Antigens

The crude antigen (CA) was obtained by sonicating the RM6/66 strain of *B. canis* using a previously described method (de Oliveira *et al*., 2011); briefly, 2 g of heat inactivated (75 °C for 1 h) *B. canis* biomass were taken and placed in 10 mL of a solution containing 10 mM HEPES pH 7.5, and store at -20 °C until use. Bacteria were thus sonicated (Vibra Cell VCX 130 - Sonics) (4 pulses of 1 min at 15 Hz) in presence of protease inhibitor cocktail (Sigma-Aldrich, Saint Luis, Mo). To corroborate cell disruption, a Gram stain was performed. Once the bacterial biomass had been sonicated, it was centrifuged at 700 x g for 30 min at 4 °C in order to obtain the soluble fraction (supernatant).

The purified protein (GroEL) was obtained from the soluble fraction by fast protein liquid chromatography (FPLC) using an anion exchange column (HiTrap Capto Q; GE Healthcare) with liquid chromatography (AKTA Pure; GE Healthcare). The column was buffered with 5 mL of a solution of Tris-HCl (5 mM) and EDTA (10 mM), then the sample was introduced with a flow range of 1 mL/min and washed with the previously described solution. The elution buffer (1 M NaCl) was included in a linear gradient from 0 % to 100 % in nine volumes of 1 mL each. Each fraction was obtained in a volume of 0.5 mL and was resolved by 12 % SDS-PAGE by duplicate and either stained with Coomassie Blue or transferred onto a PVDF membrane for immunoblotting, using sera from infected dogs; further dog anti IgG as secondary antibody (1:10000) and ECL West femto as substrate (ThermoFisher, Carlsbad, California) were used. Subsequently, fractions were concentrated 3 times in an Amicon ultra 50kDa (Millipore, Merck KGaA, Darmstadt, Germany) and resuspended in PBS and two fractions containing two bands each (between 55 kDa and 70 kDa), were selected to resolve them on a gel using isoelectric focusing (IEF) (a total of 20 µg of protein) followed by two-dimensional polyacrylamide gel electrophoresis and the antigenicity of each spot was verified by Western blotting with the aid of sera from *B. canis* infected dogs. Three spots were identified as GroEL by liquid chromatography–mass spectrometry as described in a previous work, with a coverage of 29, 51 and 21 % for each spot (Morales Aguilar, 2016) (Supplementary material).

iELISA protocol using the sonicated crude antigen

The ELISA using crude antigen (CA-iELISA) was done on 96-well mid-binding polystyrene plates (Nunc-Immuno Micro Well MaxiSorp; Sigma-Aldrich), adding 240 ng of antigen diluted in 100 µL per well in phosphate buffer solution (PBS); this was homogenized at 100 rpm for 5 min and incubated at 37 °C for 17 +/- 1 h. It was washed three times with 200 µL of a solution of PBS + Tween 20 (Tween 20; Sigma-Aldrich) at 0.1 % (PBS-Tween 20). A solution of bovine serum albumin (Bovine serum albumin; Sigma-Aldrich) diluted to 1 % in PBS (100 µL per well) was added, then incubated for 60 min at 37 °C and washed three times with 200 µL of Tween 20 at 0.1 %. The serum diluted in PBS 1:250 in 100 µL was added, then incubated at 37 °C for 60 min, after which it was washed three times with 200 µL of Tween 20 at 0.1 %. A 100 µL solution of the rabbit anti-canine IgG conjugated with horseradish peroxidase secondary antibody (Rabbit anti-Canine IgG (H+L) secondary antibody, HRP; Invitrogen), diluted 1:2000 in PBS, was added (ABTS solution; Roche), incubated at 37 °C for 60 min, and washed three times with 200 µL of Tween 20 at 0.1 %. Finally, 100 µL of ABTS were added (Sigma-Aldrich) to each well in the dark and incubated with the well covered with aluminum foil, at room temperature in orbital agitation at 100 rpm for 25 min. The plates were read using an ELISA reader (Biotek Instruments) at a wavelength of 405 nm using the program Gen5 (https://www.biotek.es/es/products/software-robotics-software/gen5-microplate-reader-and-imager-software/).

iELISA protocol using GroEL

For the technique using GroEL as the capture antigen (GroEL – iELISA), a 96-well plate (Nunc-Immuno Micro Well MaxiSorp; Sigma-Aldrich) was sensitized using 10 ng of purified GroEL protein diluted in sufficient carbonate buffer solution (0.05 M, pH 9.6) to reach 100 µL volume per well. The plate was placed in orbital agitation at 100 rpm for 5 min, covered with parafilm (Parafilm M; Merck) and incubated at 4 °C for 17 ± 1 h, then washed three times with 200 µL of Tween 20 at 0.1 %. 100 µL of serum diluted 1:500 in PBS with bovine serum albumin at 1 g/L (0.1 M, pH 7) was added, covered with parafilm, incubated for 1 h at 37 °C, and washed three times with Tween 20 at 0.1 %. 100 µL per well of a 1:2000 dilution of the horseradish peroxidase goat anti-canine IgG antibody (Goat anti-Canine IgG (H+L) secondary antibody, HRP; Invitrogen) diluted in PBS (0.1 M, pH 7), was added, covered with parafilm and incubated for 1 h at 37 °C, then washed three times with Tween 20 at 0.1 %. Finally, 100 µL of ABTS was added to each well in the dark, covering the plate with aluminum foil and placing it in orbital agitation at 100 rpm for 25 min. The reading was taken on an ELISA reader (Bio-Tek/ELX808; Biotek) at a wavelength of 405 nm using Gen5 software.

In-house validation protocol

The cutoff point was determined using a previously described method (Frey *et al*., 1998) and calculated using the following equation:

$$CUTOFF\ POINT = \bar{X} + SD\ t\sqrt{1 + (1/n)}$$

Where $\bar{X}$ is the average of the results obtained from a series of negative controls, *SD* is the standard deviation of that series of results, *t* is the percentile (1 – α) of a one-tailed Student's t distribution with (*n – 1*) degrees of freedom, and *n* is the number of controls tested. The results are considered positive for *B. canis* infection if they are above the result of this calculation. Next, precision was evaluated by calculating intermediate precision and repeatability using the supplemental material from a guide, (Andreasson *et al*., 2015) where the Coefficient of Variation (%CV) is calculated from a series of independent measurements on different days (Intermediate Precision) as well as a series of simultaneous measurements (Repeatability). In addition, the mean and standard deviation of these parameters were calculated, using a positive control and a negative control. Repetitions were done on five different days, five times each. The robustness of the tests was evaluated for positive and negative sera, varying by +/- 5 °C the incubation temperature of the reference sera, using 37 ºC as a reference temperature. Similarly, the incubation time was varied by +/- 5 min compared to the standard; the results were compared using an ANOVA and significant differences were determined by Tukey's Honestly Significant Difference (HSD) test (Andreasson *et al*., 2015). Selectivity was evaluated using sera from dogs positive for diseases other than canine Brucellosis. To evaluate the sample stability, 19 aliquots were prepared from a positive control and a negative control and were subjected to a freeze-thaw process for comparison using the following protocol: (Andreasson *et al*., 2015)

1. Aliquots 1 to 6 were thawed at room temperature for 2 h then refrozen for 12 h.
2. Numbers 2 through 6 were thawed for 2 h and refrozen for 12 h.
3. Numbers 3 through 6 thawed for 2 h and refrozen for 12 h.
4. Numbers 4 through 6 thawed for 2 h and refrozen for 12 h.
5. Numbers 5 and 6 thawed for 2 h and refrozen for 12 h.
6. Aliquot 6 was thawed for 2 h. and frozen again for 12 h.
7. Samples 7 to 12 were kept at room temperature from the start of the evaluation; one aliquot was moved to the freezer after 1 h, a second aliquot after 2 h, a third aliquot after 4 h, a fourth aliquot after 24 h, a fifth aliquot after 72 h, and the sixth aliquot after 168 h.
8. Aliquots 13 to 18 were kept at 4 °C from the start of the evaluation and were moved to the freezer using the same process as described in point 7 above.
9. Aliquot 19 was kept at –20 °C and then tested one month later.

Finally, sensitivity and specificity were tested using 10 negative and 10 positive sera, adjusting the results according to the previously described formula to obtain the 95 % Confidence Interval ($IC_{95\%}$) (DasGupta *et al*., 2001; Jacobson, 1998), using the following calculation:

$$A = 2r + 1.96^2$$
$$B = 1.96\sqrt{1.96^2 + 4r(1-p)}$$
$$C = 2(n + 1.96^2)$$

Constructing the confidence interval as *(A-B)/C, (A+B)/C.*

RESULTS

I-ELISA using sonicated crude antigen

The cutoff point at 0.98 DO was calculated using 10 negative controls. In the precision test, we found that for the negative control the CV was 3.7 % for repeatability and 13 % for intermediate precision, with a mean of 0.5 DO; for the positive control, CV was calculated as 1.1 % for repeatability and 3.8 % for intermediate precision, with a mean of 3.0 DO.

With respect to the ANOVA results for robustness, there were significant differences ($p < 0.05$) when temperature was varied, however, the Tukey's HSD test showed that the difference was between results from the incubation of serum at 32 °C and 42 °C, not between either of these and the standard temperature of 37 °C; the ANOVA of the results of tests varying incubation times showed significant differences ($p < 0.01$), which the Tukey HSD test showed to be between the incubation times of 55 min and 65 min, as well as each of these times compared to the standard 60 min.

The selectivity test allowed the identification of a false positive test using serum from dogs infected with *L. interrogans.* The sample stability test showed significant variation in the negative sample starting at 4 h at 25 °C, 24 h in refrigeration (4 °C), and the second freeze-thaw cycle, but there was no variation in the sample that was kept frozen (-20 °C) for 30 days. In the positive control aliquots, there was no significant variation during the whole procedure. The test showed a sensitivity of 100 % ($CI_{95\%}$ 70 – 100 %) and specificity of 100 % ($CI_{95\%}$ 70 – 100 %).

I-ELISA using GroEL

The cutoff point was calculated as 1.228 DO using 10 negative controls. In the precision test, for the negative control the %CV was 4.6 % for repeatability and 12.9 % for intermediate precision, with a mean of 0.7 DO. For the positive control, the %CV was calculated at 8.1% for repeatability and 16.8 % for intermediate precision, with a mean of 1.3 DO. The ANOVA for robustness showed significant differences ($p < 0.01$) when temperature was varied, which the Tukey HSD test identified to be between the results of sera incubated at 42 °C and the standard temperature (37 °C) and between incubation at 32 °C versus 42 °C. With respect to the tests where incubation time was varied, the ANOVA showed significant differences in results between 55 and 60 min, 60 and 65 min, and 55 and 65 min according to the Tukey HSD test.

The selectivity test allowed the identification of a false positive test of serum from dogs infected with *L. interrogans* and *Salmonella* spp. The test of sample stability showed that for negatives, significant differences began to occur at 168 h at 25 °C, one week in refrigeration, and the second freeze-thaw cycle, while for the positives, there were no differences, even after 168 h at 25 °C, 24 h in refrigeration, and 7 freeze-thaw cycles. However, in both cases there was a curve with significant variations during the first hours of sample storage, both at room temperature and in refrigeration. Neither positives nor negatives varied when samples were stored in a frozen state for up to a month. The test showed a sensitivity of 44 % ($IC_{95\%}$ 18 – 73 %) and specificity of 67 % ($IC_{95\%}$ 35 – 88 %).

DISCUSSION

Canine Brucellosis is a disease whose potential epidemiological risk has been underestimated, (Hensel *et al*., 2018; Hubbard *et al*., 2018; Hull and Schumaker, 2018; Krueger *et al*., 2014) resulting in little research interest. It is therefore necessary to develop diagnostic methods so that this problem can be addressed, and in this respect serological methods play an important role (Avijgan *et al*., 2019). In this study, two different antigens from *B. canis* were evaluated to their limits in two different immunoassays to establish the range conditions under which they serve to detect the antibodies present in a sample, and therefore, their validity as diagnostic tests, (Andreasson *et al*., 2015) guaranteeing that they can be applied in the field.

The first aspect that must be determined is the cutoff point, since this is used as a reference for differentiating between positive and negative samples during the rest of the tests, as well as the variations in the results. The method used it is based on probabilistic calculations, which has been preferred over the empirical method (Frey *et al*., 1998) since it offers increased certainty when making decisions about the positivity of a sample. Then, an evaluation of the precision was done based on the calculation of the repeatability and intermediate precision. According to literature, the intermediate precision should not exceed the repeatability doubled, (Andreasson *et al*., 2015) however, this rule can be

modified depending on the nature of the method being validated. For this reason, in order to consider whether the technique is reproducible, an intermediate precision and repeatability %CV below 20 %, were used as reference points (Jacobson, 1998). Under this criterion, both CA – iELISA and GroEL – iELISA have shown acceptable levels of repeatability and intermediate precision for both positive and negative samples, suggesting that the results can be confided in.

When evaluating the robustness of the CA – iELISA test, we found that the samples incubated at different temperatures did not show statistically significant differences compared to the standard; this variable refers to the interference that a sudden change in temperature inside the incubator may occur, and therefore the need for a good calibration and stability of the equipment. This was not true of varying incubation time, where differences were found. It is therefore necessary that the operator is attentive to the incubation times when using this test for diagnosis. In the case of GroEL – iELISA, there were significant differences when temperature was increased, as well as when incubation time was modified, so we recommend that before carrying out the test, the operator ensures that the temperature of the equipment is kept above 37 °C and below 42 °C, as well as adhering to the proposed incubation times. This variable is totally operator-dependent.

When evaluating the selectivity of the tests, it was found that the CA – iELISA test showed cross-positivity with dogs infected with *Leptospira* spp. In the GroEL – iELISA test, there was an additional cross-reaction with *Salmonella* spp. The cross-reaction with *Leptospira* spp. has been described previously in other serological methods (Rose Bengal) in cattle vaccinated against this spirochete, (de Faria Naves *et al*., 2012) furthermore, this has been reported in dogs by personal communication (Moreno-Torres A, 2018). Moreover, in the only literature specifically addressing this question, no cross-reaction was found between *B. canis* and *Leptospira* spp. using the RSAT test (Krecic, 2019). This study is therefore the first to demonstrate a cross-reaction between *B. canis* and *Leptospira* spp. Due to the nature of the capture antigens used as a reference, and the lack of positive reactions to them in a method based on the detection of membrane antigens, we can infer that the antigens responsible for the interference are of cytosolic origin. This chaperone protein is expressed abundantly in biofilms of *Leptospira* spp.(Vinod Kumar *et al*., 2017) which suggests a possible relationship between the formation of these biofilms and the high exposure to GroEL that generates antibodies against these molecules, though this question is beyond the scope of this study. In addition, it has been found that GroEL from *Salmonella* spp. is highly homologous to GroEL from *Escherichia coli*, and both are highly homologous to a protein present in *Brucella* spp., (Panchanathan, 1998; Sekhavati *et al*., 2015) which could explain the cross-reaction in the GroEL – iELISA test with *Salmonella* spp. To answer this question, it is necessary to address the problem from a molecular or bioinformatics perspective to determine the similarities between GroEL of the two species

of bacteria. There are other pathogens that are known to cause cross reactions with *Brucella* spp. (Mol *et al*., 2020), those possible interference in the diagnosis should be studied in the future to rule out cross reactions. One of the best known is the case of *Yersinia enterocolitica*, which interferes *with B. melitensis* and *B. abortus* detection; however, to date there is no published data that indicates that this pathogen affects different *B. canis* serological tests. Instead, Hurvell in 1972 demonstrated that *Yersinia enterocolitica* antigens does not cross-react with rough *Brucella*, namely *B. canis* and *B. ovis* (Hurvell, 1972).

Based on the stability test results, it is recommended that samples for CA – iELISA be kept at 25 °C for less than four h, in refrigeration for less than 24 h, with freezing being the preferred sample storage method, since reliable results were yielded even after one month of freezing. For the GroEL – iELISA, it is recommended that samples be left no more than one h at room temperature or in refrigeration and that they be placed immediately in a freezer to ensure that the results are reliable.
The sensitivity and specificity calculated for CA – iELISA are good, but the confidence interval is too lax because of the number of samples used, so they should be interpreted with this in mind. The same is true for GroEL – iELISA. Since for the “in-house” validation of serological tests, the determination of sensitivity and specificity is not required according to the method described by Andreasson (Andreasson *et al*., 2015), in this work a larger number of samples was not included; instead, we decided to calculate these parameters with the sole purpose of obtaining a point of comparison between the tests that brings us closer to possible scenarios when using it in daily clinical practice. In future work, more samples should be analyzed to allow more precise estimation of the parameters, in order to use some of these tests for epidemiological studies. Since that was not the objective of this study, we did not explore this issue further.

Finally, the comparison between the tests presented in this study, reinforces the idea that the use of diverse antigens in a diagnostic test is preferable to the use of a single antigen (Wanke *et al*., 2002). Nonetheless, the discussion of whether cytosolic antigens are more useful in diagnosis than membrane antigens remains open, since better performance was observed in the test that used a mixture of antigens of both origins than the test that used cytosolic antigen alone.

Canine brucellosis is a disease that requires more attention and research. Thus, the development of diagnostic methods with adequate quality control will allow future investigations that explore more deeply the impact of this disease on human and animal health and lead us to the improvement of prevention and control strategies following the One Health philosophy.

CONCLUSIONS

Considering the points above, it can be concluded that CA – iELISA not only performed better than GroEL – iELISA, but also that, given the results obtained, it is a useful diagnostic test for canine brucellosis in the field. Since it detects not only external membrane antigens, but also cytosolic antigens, CA – iELISA is more diagnostically useful than immunoagglutination tests. CA – iELISA also allows us to address the immune response of the host semi-quantitatively (not just qualitatively), making it potentially useful for the first contact veterinarian monitoring the patient serologically during treatment.

ACKNOWLEDGEMENTS

We thank Dr. Díaz-Aparicio Efrén for kindly commenting on the final version and Moreno-Torres Azaf for the personal communication regarding the cross-reaction between *Brucella* spp. and *Leptospira* spp. Victor Palacios-Rodríguez received a scholarship from the Mexican National System of Researchers (SNI), CONACYT.

FUNDING

This research work was financed by DGAPA-UNAM, PAPIIT project No. IT201017.

CONFLICTS OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Supplementary material Figures 1-4 show the GroEL protein purification process. Table 1 corresponds to protein identification by liquid chromatography-mass spectrometry.

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