

## Characterization of Bovine *Brucellosis* in Korean Native Cattle by Means of Immunohistochemistry and Proteomics

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This study was conducted to examine the utilization of immunohistochemistry using the bovine anti-brucella immunoglobulin G (IgG) antibody in the diagnosis of brucellosis and to develop a functional biomarker relation for the progress of the disease. Anti-brucella IgG antibody was purified from the affected bovine serum using an affinity chromatography. We performed our investigation on 17 cases of brucellosis and 19 control cases with negative Rose-Bengal test results. Our purified anti-brucella IgG antibody showed a positive immunoreactivity in cytoplasmic hepatocytes of the centrilobular region, and glomeruli and tubular epithelium of the kidney. The protein pattern of the affected liver versus control was analyzed by two-dimensional electrophoresis, showing a different expression pattern of proteins between the two. Five protein spots were up-regulated and another were five down-regulated in the brucellosis liver. Significant upregulation of catalase and 3-hydroxyacyl-CoA dehydrogenase might be due to a compensatory reaction in response to the endotoxic shock of brucella. In conclusion, the anti-brucella IgG antibody may be a good tool for discriminative diagnosis of the affected tissues and proteomics data suggest new target proteins underlying a possible pathogenic mechanism of brucellosis.

**Key words** : Anti-brucella IgG, bovine, Brucellosis, immunohistochemistry, proteomics

### Introduction

Brucellosis is primarily a reproductive disease characterized by abortion, retained placenta and impaired fertility in the principal animal host [30]. Eight species have been identified within the genus, namely, *Brucella abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis*, *B. suis*, *B. cetacea*, and *B. pinnipedia* [7,18,27]. *Brucella* species are gram-negative coccobacilli as facultative intracellular parasites, which resist intracellular killing and replicate a niche in professional and non-professional phagocytosis [11], without spore forming, motility, and encapsulation. *Brucella* species are easily aerosolized, highly invasive and extremely infectious, and recognized as potential agricultural, civilian and bioterrorism hazards [28]. Even though brucellosis might be an infectious disease with variable diagnostic methods in veterinary medicine, so far there is no sensitive and specific diagnostic method without non-specific result. It is difficult to find out the *brucella* infection because the affected animal shows no clin-

ical signs except abortion and sterility. As far as latent infection, brucellosis may mimic many other diseases. The limited clinical presentation and histopathological findings could be difficult to diagnosis and prognosis of brucellosis in animal and human being due to most of affected animals were burying. Thus we investigated the pathological and immunohistochemical examination using purified bovine anti-brucella IgG. Furthermore, we also performed proteomic analysis for the infected livers, which may lead to characterization of functional proteins in regarding pathogenesis and progressing of brucellosis.

### Materials and Methods

#### Blood and tissue samples

All blood and tissue samples were collected from Korean native cattle in Gyeongbuk province. Of the total thirty-six heads, seventeen heads showed positive reactions to *B. abortus* by both Rose-Bengal test (RBT) and Tube agglutination test (TAT), which is presented to necropsy and collected blood and tissues for positive case. Nineteen serological negative heads, as control group, samples were collected from the car-

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casses at the slaughter house as a separate set. Whole and/or heparinized blood samples were collected, then centrifuged and separated into serum and plasma, respectively, and presented to the RBT and TAT. Collected tissues for a histopathologic examination were salivary gland, liver, pancreas, spleen, uterus (contain with placentome, cotyledon and inter-cotyledon placenta), intestine, heart, lung, supramammary lymph nodes, skeletal muscle, fetal lung and kidney. The livers were frozen at  $-70^{\circ}\text{C}$  for proteomic analysis.

#### Histopathology

All tissue pieces from each Korean native cattle were rapidly removed and fixed in a 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections were cut to 4-6  $\mu\text{m}$  in thickness. The sections were stained with hematoxylin and eosin (H-E).

#### Purification of bovine anti-brucella IgG antibody

Generally, we followed by based on the manufactured protocol as follows. Brucella antisera obtained from brucella positive case based on RBT and TAT were filtered for clearing and allow to equilibrate to experimental temperature ( $2-8^{\circ}\text{C}$ ). A column coated with recombinant protein G agarose, affinity chromatography, was used for concentration of the IgG to be purified. Then purified bovine anti-brucella IgG was frozen for the immunohistochemistry.

#### Immunohistochemistry

Sections were deparaffinized in xylene, rehydrated in graded alcohol series, incubated in a solution of 3% hydrogen peroxide in methanol for 30 min and microwaved at 750 W for 10 min in 10 mmol/l citrate buffer, pH 6.0. Tissue sections were washed with PBS (phosphate buffered solution, and then immunostained with primary antibody, bovine anti-brucella IgG, previously described purification. The antigen-antibody complex was visualized by an avidin-biotin-peroxidase complex (ABC) solution using an Histostin-plus Bulk kit (Zymed Laboratories, Inc., USA) with 3, 3-diamino benzidine (Zymed Laboratories, Inc., USA). Tissue sections were then rinsed in distilled water and counterstained with Mayer's hematoxylin. For negative control, the primary antibody was replaced by PBS.

#### Separation of Brucella affected liver proteins by 2D SDS-PAGE gel analysis

To identify proteins expressed in the *Brucella* affected liv-

er, proteins were analyzed by 2-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) and MALDI-TOF. Proteins were extracted from livers of *Brucella* negative (control) and *Brucella* positive cases proven in the serum RBT and immunohistochemistry, following homogenization in IEF (isoelectric focusing) sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTE, 2% IPG buffer, and 3.6% protease inhibitors) by sonication (26 for 20 sec each time). The suspension was centrifuged at 13,000 rpm for 30 min, supernatants were collected, aliquoted, and stored at  $-80^{\circ}\text{C}$  until usage. Protein concentration was estimated by the use of Bradford reagent (BioRad, Hercules, CA., USA).

#### 2-DE

2-DE was performed as previously described [17,25] with the following specifications: For the IEF, 18 cm nonlinear strips, pH range of 3-10 (Amersham Bioscience, Sweden) were used. The strips were placed in rehydration buffer (7 M urea, 2 M Thiourea, 4% CHAPS, 65 mM DTE, 0.2% IPG buffer, 400 mM Tris) for 13 hr before the IEF. After strip rehydration a sample volume containing 750  $\mu\text{g}$  of total protein was diluted with IEF sample buffer to a final volume of 250  $\mu\text{l}$  and loaded on the strips using cups. IEF was performed according to the following steps: 500 V for 1 hr, 1,000 V for 1 hr, 1,000-8,000 V for 2,000 Vh and 8,000 V for 32,000 Vh. Strips were then applied on the top of 12% polyacrylamide gels and SDS-PAGE was performed for 20 min at a constant current of 20 mA per gel and then 40 mA per gel until the Bromophenol blue reached the bottom of the gels. Gels were stained with Coomassie Colloidal Blue (Novex) and scanned at a PowerLook 1120 (UMAX systems GmbH, Germany). Electronic images of gels were analyzed using the ImageMaster 2D software (Amersham Biosciences). After global normalization, reproducible spots from among triplicate experiments were analyzed quantity.

#### MALDI-TOF MASS

Protein spots were cut horizontally between molecular mass marker and transferred to microcentrifuge tube. Stained gel pieces were destained and the SDS removed by washing three times for 1 hr in 100  $\mu\text{l}$  1:1 acetonitrile/water. Gel pieces were dried for 30 min under vacuum and rehydrated with sequencing grade trypsin. Enzymatic digestion was performed with the addition of 20  $\mu\text{l}$  of 0.0125  $\mu\text{g}/\mu\text{l}$  sequence grade modified trypsin (Promega, Madison, USA) in 50 mM

ammonium bicarbonate and 5 mM calcium chloride, and then incubated at 37°C for 16 hr [25]. In brief, peptide masses were determined by Matrix-assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF/TOF MS) (Voyager-DE STR, Applied Biosystem, CA,USA). Spectra were calibrated upon acquisition using a ProteoMass Peptide MALDI-MS Calibration Kit, MS-CAL2 (Sigma, St. Louis, USA). The database searches were carried out with MS-Fit. (<http://prospector.ucsf.edu>). Stringent criteria were used for protein identification with a maximum allowed mass error of 25 ppm and a minimum of four matching peptides.

## Results

### Histopathology and Immunohistochemistry

Most of tissue sections for the positive serologic test showed mild to moderate parenchymal degeneration and diffuse hyperemia. Sinusoidal hyperemic liver tissue in H-E staining showed positive immunoreactivity for the purified anti-brucella IgG predominant in the centrilobular hepatocytes. These immunoreactivity corresponded to relative intensity from 1+ to 2+ in the cytoplasm (Fig. 1). Kidney showed slight hyperemia in the parenchyma similar to the liver. In the cortex and medulla lesion revealed positive for the purified anti-brucella IgG, especially tubular epithelium had strong immunoreactivity. In the distal convoluted tubules of the kidney showed highly immunoreactions to the anti-brucella IgG. Among the presented tissues, the 9-months-

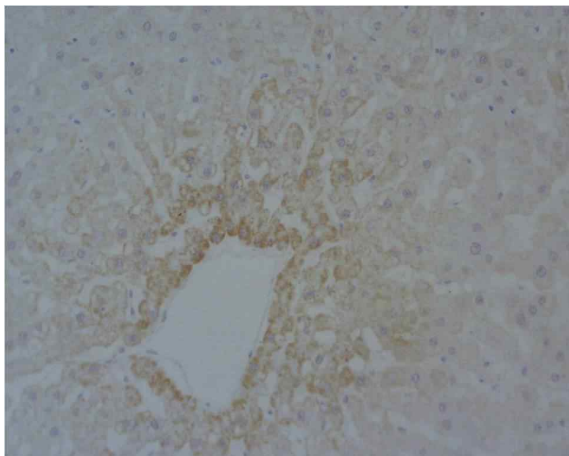


Fig. 1. Immunoreactivity against bovine anti-brucella IgG from brucella antisera in liver section. Strong immunoreactivity was seen in hepatocytes of the centrilobular area of the liver. Original magnifications:  $\times 400$ .

old still-born fetus showed strong immunoreactivities in the glomeruli and undifferentiated tubular epithelium of the kidney (Fig. 2). Other histopathological findings were hyperemic pneumonia, collapsed and or thickened alveolar walls, and hypertrophy of peribronchial smooth musculature (Fig. 3). These lungs revealed positive immunoreactivity in the bronchiolar epitheliums at a high magnification (Fig. 4). In the uterine of the still-born fetus, immunoreactivities revealed cytoplasmic expressions in the epithelium of a glandular lesion (Fig. 5).

The control tissue sections showed similar histopathological features of liver, kidney, and lung in the H-E staining. However, no immunoreaction observed in all tissue section

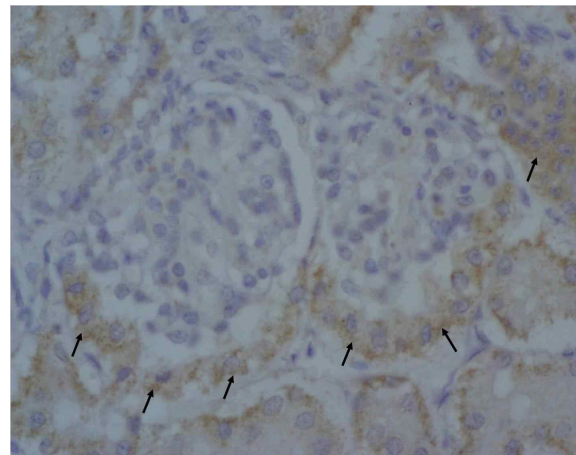


Fig. 2. Immunoreactivity against bovine anti-brucella IgG in kidney section from the 9-months-old still-born fetus. Strong immunoreactivity (arrows) was seen in the glomeruli and tubular epithelium of the kidney. Magnification  $\times 400$ .

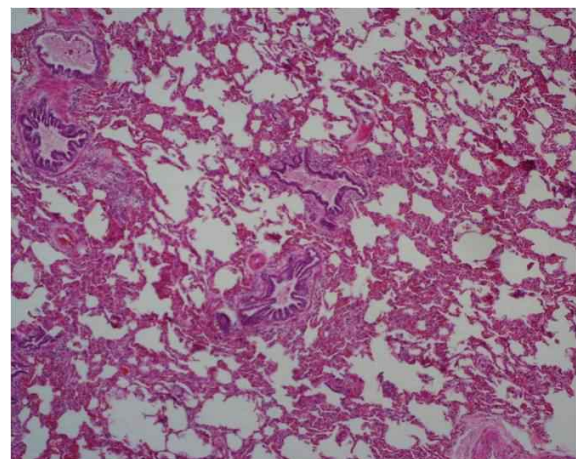


Fig. 3. Hyperemia and collapses of alveolar walls were present and mild pneumonia in some cases. H-E staining. Magnification  $\times 100$ .



Fig. 4. Immunoreactivity against bovine anti-brucella IgG in lung section. Immunoreactivity (arrows) was shown in the bronchiolar epitheliums. Magnification  $\times 400$ .

including intestine, heart and skeletal muscle for the purified anti-brucella IgG. In the conclusion, taken by all the results of immunohistochemistry immune reactions were observed in 7 in the kidney and liver, and in 3 in the lymph node among the 17 serologic brucella positive cases (Table 1). All of the 19 brucella negative cases revealed no immune reactions.

Characterization of possible pathogenic proteins

The hepatic proteomes of the brucella positive and control cases by using 2-D gel electrophoresis and MALDI-MS from

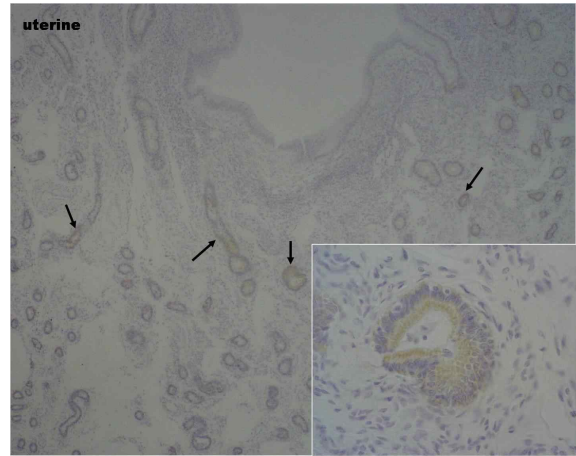


Fig. 5. Immunoreactivity against bovine anti-brucella IgG in uterine section from the 9-months-old still-born fetus. Immunoreactivity (arrows) was shown in cytoplasm of the epithelium in the glandular of uterine; the inset in the lower right corner is glandular lesion of uterine. Magnification  $\times 100$ , the inset  $\times 400$ .

each the triple analysis. The individual protein spot quantity was normalized as a percentage, and a comparison between two groups was assessed using a Student's *t*-test. The protein spots were detected between pH 3 and 10. Of the ninety-four observed spots, sixty-five proteins were identified by MALDI-MS spectrometry. The average molecular weight value of all the detected spots was 43.21 kDa and the average pI was 6.57. Five proteins showed expressions, which were

Table 1. Immunohistochemistry by using purified anti-brucella IgG (Brucella positive cases)

Animal I.D. (sex/age)	Kidney	Liver	Spleen	Heart	Lymph node	Intestine
B050407-1 (♀/1)	+	+	-	-	-	-
B050407-2 (♀/2)	-	+	-	-	-	-
B050407-3 (♀/2)	++	+	-	-	-	-
B050407-4 (♀/2)	-	-	-	-	-	-
B050407-5 (♀/2)	+	+	-	-	-	-
B050413-1 (♀/4)	++	-	-	-	-	-
B050413-2 (♀/3)	-	+	-	-	++	-
B050413-3 (♀/2)	-	+	-	-	++	-
B050413-4(9 mo. fetus)	++	-	-	-	++	-
B050413-5(7 mo. fetus)	++	-	-	-	-	-
B050517 (♀/2)	-	-	-	-	-	-
B050519-1 (♀/5)	-	-	-	-	-	-
B050519-2 (♀/3)	+	+	-	-	-	-
B050519-3 (♀/5)	-	-	-	-	-	-
B050604-1 (♀/1)	-	-	-	-	-	-
B050604-2 (♂/1)	-	-	-	-	-	-
B050604-3 (♀/1)	-	-	-	-	-	-

Relative intensity of immunoreactivity against IgG fractions from brucella antisera was graded from 1+ to 2+ in stained cytoplasm. Negative (-); no cells were stained.

Table 2. Proteins expressed by up-regulation in the liver of Brucella positive cases compared with control animals

Spot No.	protein name	Accession No. <sup>a</sup>	MW(kDa)/pI <sup>b</sup>	Relative Ratios <sup>c</sup>
1	Eukaryotic initiation factor 4A-III (EC 3.6.1.)	Q2NL22	46841/6.3	3.36
2	Annexin A4 (Annexin IV)	P13214	35889/5.5	3.01
3	Catalase (EC 1.11.1.6)	P00432	59916/6.8	2.48
4	Catalase (EC 1.11.1.6)	P00432	59916/6.8	2.87
5	3-hydroxyacyl-CoA dehydrogenase type-2 (EC 1.1.1.35)	O02691	27141/8.4	2.00

<sup>a</sup>Protein No. of SwissProt database (2009.09.25).

<sup>b</sup>Obtained from the MS fit search of proteinprospector database.

<sup>c</sup>Relative % volume of spot density in liver of brucellosis bovine vs. liver of control bovine.

Table 3. Proteins expressed by down-regulation in the liver of Brucella positive cases compared with control animals

Spot No.	Protein name	Accession No. <sup>a</sup>	MW(kDa)/pI <sup>b</sup>	Relative Ratios <sup>c</sup>
1	Origin recognition complex subunit 4	Q2YDI2	50328/8.1	0.37
2	Histidine-rich glycoprotein (Histidine-proline-rich glycoprotein) (HPRG)	P33433	44471/7.1	0.16
3	Guanidinoacetate N-methyltransferase (EC 2.1.1.2)	Q2TBQ3	26611/5.7	0.36
4	Ubiquitin-conjugating enzyme E2 S (EC 6.3.2.19)	Q1RML1	23901/8.8	0.37
5	Protein disulfide-isomerase precursor (EC 5.3.4.1)	P05307	57266/4.8	0.49

<sup>a</sup>Protein No. of SwissProt database (2009.09.25).

<sup>b</sup>Obtained from the MS fit search of proteinprospector database.

<sup>c</sup>Relative % volume of spot density in liver of brucellosis bovine vs. liver of control bovine.

with up-regulated (Table 2), and another five proteins were down-regulated (Table 3).

## Discussion

The wide distribution and prevalence of prevalence of bovine brucellosis in organized farms warrants immediate attention and suitable preventive diagnosis should be developed and implemented. Identifying infected animals and removing from the herd is key control of brucellosis in both the livestock and the human populations throughout the world. The employed methods of diagnosis of bovine brucellosis are culture, the complement fixation test, serum agglutination test, Rose-Bengal test (RBT), indirect enzyme-linked immunosorbent assay and the fluorescent polarisation assay presently.

Brucellosis was first recognized as a disease affecting humans on the island of Malta in the 19th and early 20th centuries [23]. In Korea, more than 200 cases of brucellosis in humans [16], and about 4,400 outbreaks in bovines were reported [2]. Clinical manifestations of bovine brucellosis depend on age, reproductive and immunological status, route of infection, and virulence and dose of the *Brucella* strain [1]. Seropositive animals were slaughtered and buried because of eradication program employed in numerous

countries throughout world. There are gross lesions and microscopic changes of various infected organ and tissue were limited data without experimental brucellosis [21] and described goat and sheep [3,10,19-21]. The present study we examined the pathological, immunohistochemical examination of brucellosis not for the experimentally infected animal model but the field infected using the purified bovine anti-brucella IgG. The most common pathologic changes of our case were consistent with previously reported [6,21]. Our purified anti-brucella IgG showed highly cytoplasmic expression in the centrilobular hepatocytes and glomeruli/ tubular epithelium in the kidney of seropositive case compared with control animals. Our immunohistochemical results showed a versatile method for localizing specific antigens in tissues or cells based on antigen-antibody recognition.

Moreover, we analyses pathogenic related proteins of seropositive and immunolabelled liver tissue. Brucella affected liver tissue revealed significantly changes of expression levels about ten proteins. Marked up-regulated proteins are eukaryotic initiation factor, annexin A4, catalase and 3-hydroxyacyl-CoA dehydrogenase type-2. Eukaryotic initiation factor 4A-III (eIF4A-III) is a nuclear protein that physically or functionally interacts with translation initiation factors 4G (eIF4G) and 4B (eIF4B), respectively, and shares strikingly

high identity with the initiation factors eIF4A-III. It binds to spliced mRNAs and is involved in nonsense-mediated decay (NMD) of mRNAs containing premature stop codons (PTC). eIF4A facilitate binding of the small ribosomal subunit [9]. Annexin A4 (Annexin IV) is believed to be involved in exocytosis and regulation of epithelial  $\text{Cl}^-$  secretion. It may play a role in alveolar type II cells through interaction with the surfactant protein SFTPA1 (SP-A). Annexins are  $\text{Ca}^{2+}$ -regulated phospholipid-binding proteins. Catalase (EC 1.11.1.6) is a heme containing redox enzyme found in high concentrations in a compartment in cells called the peroxisome [5]. Catalase can decompose hydrogen peroxide in reactions catalyzed by two different modes of enzymatic activity such as the catalytic the peroxidatic mode. Bovine liver catalase is a slightly ellipsoidal tetramer of 60 kilodalton subunits and their different crystal forms related with different crystallisation conditions. Bovine liver catalase contains four identical subunits equipped with a high-spin Fe (III) protoporphyrin IX. 3-hydroxyacyl-CoA dehydrogenase type-2 (Type II HADH, EC 1.1.1.35) was isolated from seropositive and immunolabelled liver, which was absolutely consistent with previously reported bovine liver protein [31]. It involved in mitochondrial fatty acid  $\beta$ -oxidation by catalyzing of straight chain 3-hydroxyacyl-CoAs. The enzyme was also known as amyloid  $\beta$ -peptide binding protein related to over-express in brain of Alzheimer patients [13]. Human short chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) deficiency is an inherited defect in mitochondrial fatty acid oxidation [31]. Significant upregulation of catalase and 3-hydroxyacyl-CoA dehydrogenase might be due to compensatory reaction to endotoxic shock of brucella [22]. In other words, septic shock may induce ATP depletion that may cause an increase in peroxisomal  $\beta$ -oxidation in order to produce ATP, while abundant  $\text{H}_2\text{O}_2$  may be produced during peroxisomal  $\beta$ -oxidation [12]. This  $\text{H}_2\text{O}_2$  might be scavenged by increased catalase in the brucellosis liver.

Significantly down-regulated proteins are origin recognition complex subunit 4, histidine-rich glycoprotein, guanidinoacetate N-methyltransferase, ubiquitin-conjugating enzyme E2 S and protein disulfide-isomerase precursor. Origin recognition complex subunit 4 has a role in both chromosomal replication and mating type transcriptional silencing. Histidine-rich glycoprotein (HRGP) is an abundant heparin binding plasma protein [15]. It can inhibit rosette formation and is known to interact with heparin, thrombospondin and the lysine-binding site of plasminogen. On the basis of its

homology with high molecular weight (HMW) kininogen, the His-rich region of HRGP may mediate the contact activation phase of intrinsic blood coagulation cascade. Guanidinoacetate N-methyltransferase (GAMT, EC 2.1.1.2) is catalytic enzyme involved in the last step of creatine biosynthesis [4]. The enzyme is found in abundance in the livers of all vertebrates and purified to homogeneity from the livers of pig and rat [24]. A hereditary disease with extra-pyramidal motor disorder revealed extremely low concentrations of creatine in brain, serum and urine, moreover, recently described deficiency of GAMT in the liver [26]. Ubiquitin-conjugating enzyme (E2s) catalyzes the covalent attachment of ubiquitin to other proteins. Ubiquitination is a major form of posttranslational modification of proteins in eukaryotes and generally leads to the degradation of target proteins [14]. The fifth down-regulated protein disulfide-isomerase precursor (PDI, EC 5.3.4.1) is the smaller subunit of the microsomal triglyceride transfer protein complex [15] found in the lumen of the endoplasmic reticulum which uniquely binds various peptides [8].

There was no significance difference of histopathology between seropositive animals and healthy controls. Immunohistochemistry of purified anti-brucella IgG would be excellent alternative method to examine tropism of the pathogen for the tissue in the case of no marked histopathological changes observed. Our results for the proteomics of the affected liver tissue suggested the pathogenesis related proteins. Generally changes of protein expression involved in the pathogenesis and disease occurrence. Our limited data would be noteworthy comprehensive bioinformatics resource for brucella to facilitate research of counter-measures including drugs, vaccines and diagnostics. New immunogenic proteins have been elucidated and would provide a reference map for future studies of this pathogen under intracellular-host conditions. With all these data taken together it would be necessary to apply and understand the pathological mechanism of brucellosis and furthermore our findings could be applied to screening not only affected animals but could be extended to other species.

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초록 : 면역조직 화학법 및 단백질체 변화 분석을 통한 한우에서 발생한 브루셀라증의 특성

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본 연구는 브루셀라증 감염 소 혈청으로부터 분리한 항 브루셀라 면역글로블린 항체를 이용하여 조직 면역 염색을 통한 브루셀라증 진단의 활용 가능성을 조사하고 병의 발생과 관련한 기능적 진단 마커를 개발하고자 하였다. Rose-Bengal test에 대해 양성 반응을 나타내어 브루셀라증으로 진단된 17개의 케이스와 음성 반응을 나타낸 19개의 대조군 케이스에 대해 조사를 실시하였다. 본 실험실에서 분리한 항 브루셀라 항체를 이용한 면역조직화학적 반응에서 간의 중심 소엽에 위치한 간세포의 세포질, 신장의 사구체 및 관 상피에서 강한 양성 반응을 나타내었다. 감염된 소의 간과 비감염 대조군의 간의 2차원 전기 영동법에 의한 단백질체를 비교 분석한 결과, 발현량이 대조군에 비해 유의적으로 증가한 5개의 단백질 스팟과 반대로 대조군에 비해 발현량이 현저히 감소한 5개의 단백질 스팟을 선별 하였다. 이 중 카탈라아제와 3-hydroxyacyl-CoA dehydrogenase의 발현 증가는 브루셀라증에 의한 장독 쇼크에 의한 산화적 스트레스 증가에 대한 방어적 반응으로 사료 되었다. 결론적으로, 항 브루셀라 면역글로블린 항체는 감염 조직의 감별 진단을 위한 좋은 진단 재료 임과, 더 나아가 단백질체학 분석을 통해 브루셀라증 진단 및 병리 연구를 위한 새로운 마커 단백질을 제시하였다.