Immunohistochemical and Microbiological Detection of *Brucella abortus* in Aborted Bovine Fetuses

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Abstract

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Twenty-five bovine fetuses naturally infected with *Brucella abortus* were morphologically and immunohistochemically evaluated in association with bacteriologic culture. Histopathological changes were mainly bronchopneumonia in the lungs, lymphoid hyperplasia and lymphoreticular hyperplasia in the liver and spleen. Histopathologic changes in other organs and tissues revealed hematogenous spread of the infection. Immunoreactivity to *Brucella abortus* was detected in all the lungs (25 fetuses) examined. However, the antigen was not detected in any of the thymus examined. Intracellular antigenic localization was identified mainly in macrophages, neutrophils and hepatocytes. In addition, *B. abortus* strains were isolated from abomasal contents and lungs of 22 fetuses. Eighteen of the strains were biotype 1 and the remaining four were biotype 3. These findings indicate the usefulness of immunohistochemistry in suspected cases where bacteriologic culture is negative and in cases where serology is not possible or material fixed in formalin.

Brucella abortus, abortion, bovine fetus, immunoperoxidase, biotyping

Brucellosis is an important bacterial disease of domestic animals and humans caused by members of the genus *Brucella* (Enright et al. 1984; Corner et al. 1987; Crawford 1990). *Brucella abortus* is the most common causative agent of brucellosis in cattle, while the infection with *B. melitensis* and *B. suis* is rarely seen in these animals (Fensterbank 1987). The disease is primarily characterized by abortions, premature delivery and reduction of milk production. Bovine brucellosis is still endemic in many regions of the world although it has been succesfully eradicated in some countries (Crawford 1990; Kouba 2003). Despite advances in diagnosis and therapy, brucellosis is still widespread with a high prevalence in the Kars province, Turkey, ranging from 6.5% to 74% (Demirozu et al. 1994; Seyda et al. 1997). Because subacute and chronic infections of brucellosis are an important human health hazard (Carrillo 1990), it is helpful to have a wide range of diagnostic tools for this disease as possible.

Immunohistochemical techniques have been utilized to detect the location of *Brucella* organisms in formalin-fixed, paraffin-embedded tissues of cows (Meador et al. 1986; Meador et al. 1989), goat (Anderson et al. 1986; Meador et al. 1986; Meador et al. 1988), sheep (Yazıcıoğlu 1997), and mice (Meador et al. 1986). In the present study the presence and location of *Brucella* antigens in tissue sections of naturally aborted bovine fetuses was evaluated by utilising anti-*B. abortus* polyclonal antibody using the avidin-biotin-peroxidase (ABC) immunohistochemical staining technique. The results of the immunoperoxidase staining were compared with those of bacteriologic analyses.

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Materials and Methods

Histopathology and immunohistochemistry

The study was done on tissues from 25 aborted bovine fetuses. These fetuses were submitted to the Pathology Department, University of Kafkas, Kars, Turkey for necropsy between December 1998 and February 2002. None of the herds that aborted fetuses come from vaccinated against the *Brucella abortus* infection. Tissues examined included fetal lungs, liver, heart, kidneys, spleen, brain, bronchial lymph node, thymus, and abomasum. Specimens were fixed in 10% neutral buffered formalin, processed routinely for histopatologic analysis. All sections were stained with hematoxylin and eosin.

For the immunohistochemical analysis, sections of 4 µm thickness were treated with freshly prepared 3% H_2O_2 in methanol for 15 min. to remove endogeneous peroxidase activity. After three rinses with phosphate-buffered saline (PBS), all tissue sections were incubated with 5% normal goat serum (Dako, Carpinteria, USA). An anti-*B. abortus* polyclonal antibody (Difco lab., Detroit, MI), prepared in rabbit and diluted 1:100 in PBS with 2% normal goat serum was applied overnight at 4 °C, was used. After three rinses with PBS, sections were incubated with biotinylated goat anti-rabbit immunoglobulin G (Dako, Carpinteria, USA) diluted 1:200 in PBS for 60 min at room temperature. Sections then were incubated with streptavidin peroxidase complex (ABC; Dako, Carpinteria, USA). The peroxidase was localized with DAB chromogen. Sections were counterstained with Mayer's hematoxylin, dehydrated and mounted. Control sections were incubated with normal rabbit serum instead of primary antibody.

Bacteriology

Abomasal contents and fetal lungs were cultured on 7% blood agar (Oxoid, CM 271) and *Brucella* Medium (Oxoid, CM 169) supplemented with *Brucella* Selective Supplement (Oxoid, SR209E). Cultures were incubated at 37 °C for 5 to 7 days aerobically and microaerobically (Microaerobic kit, Merck, Anaerocult C). After incubation, colonies were identified on the basis of Gram stained morphology, cultural characteristics, motility, the production of catalase, urease, oxidase and H_2S, growth in the presence of CO_2 and dyes, and agglutination with the monospecific A and M antisera. In the study, *Brucella* strains were biotyped according to the method of Ribierio and Herr (1990). Field strains were distinguished from the vaccine strain (*B. abortus* S 19) by their ability to grown on media containing penicilline (5IU/ml). Utilisation of L-arabinose and D-ribose were used to differentiate isolated strains from *B. melitensis*.

In all the cases included in this study, the immunohistochemical analysis and the microbiological isolation of *Brucella* were evaluated separately.

Results

Gross lesions

The most of abortions occurred in the seventh and eighth months of gestation. Gross lesions were present in all naturally infected fetuses. Differences in the character or distribution of lesions in these fetuses were not noticed. Their subcutaneous tissues were oedematous and their thoracic and abdominal cavities contained an excess of thin red-tinted fluid. Most of the infected fetuses had changes in lungs, abomasal contents and multiple lymph nodes.

Focal-to-diffuse, purple-to-tan, firm areas were noticed throughout affected lung lobes. In several fetuses, both caudal lobes were entirely involved. The lobes were gray, firm and enlarged with indentations from adjacent ribs. Pleural roughening and tags were seen infrequently. Lymph nodes in infected fetuses were larger than expected. Multiple nodes were involved but the bronchial lymph nodes were most consistently affected. These nodes were edematous and the cortical regions were markedly thickened and distinct. The abomasum usually contained a viscous turbid brown-yellow fluid with suspended white flecks of fibrin.

Histopathology

Results of the histopathologic, immunohistochemical and bacteriologic analyses are shown in Tables 1 and 2. Histopathological changes were routinely seen in the fetal lungs. Lesions were also observed in the parenchymatous and other fetal organs.

Liver: 48% of the fetuses had no hepatic lesions. However, infected fetal livers (12 of 25) had diffuse reticuloendothelial cell hypertrophy. All of the affected fetuses had randomly scattered mild to moderate, periportal, mononuclear cell and/or neutrophil infiltration and intrasinusoidal granulomatous nodules. Eleven of affected fetuses had mild to severe hydropic degeneration in the hepatic parenchyma.

Table 1
Comparison of histopathological findings and immunohistochemical results in parenchymatous organs and bacteriological culture of aborted bovine fetal abomasal contents

	Culture	Histopathological lesions*								Immunohistochemistry**				
Fetus	*Brucella*	Liver			Lungs		Spleen	Kidney	Heart	Liver	Lungs	Spleen	Kidney	Heart
No.		PPLI	ICA	HD	BP	BIP								
1	+	-	-	-	+++	-	++	-	-	-	+++	++	-	-
2	+	++	++	++	+++	-	-	+	+	+	+++	+	-	-
3	+	++	+	+	-	+++	+	++	+	-	+++	-	-	+
4	+	++	++	++	+++	-	-	++	+	-	+++	++	+	+
5	+	++	++	++	+++	-	+	-	-	+	+++	++	-	-
6	+	-	-	-	-	++	-	-	-	-	+++	-	-	-
7	+	-	-	-	-	++	-	-	+	-	++	-	-	-
8	+	-	-	++	+++	-	-	-	-	-	+++	-	-	-
9	-	-	-	-	+++	-	-	-	-	-	+++	-	-	-
10	+	++	++	++	++	-	-	-	-	++	++	-	-	-
11	+	-	-	-	++	-	-	++	-	-	++	-	+	-
12	+	-	-	-	++	-	-	-	+	-	++	-	-	+
13	+	-	-	-	+++	-	-	-	-	-	+++	-	-	-
14	+	++	++	+++	-	+++	+++FN	-	+	++	+++	+++	-	-
15	+	++	++	-	+++	-	+	-	-	+	+++	+	-	-
16	+	++	++	-	+++	-	-	-	-	+	+++	-	-	-
17	+	-	-	-	+++	-	-	-	-	-	+++	-	-	-
18	+	++	++	+++	+++	-	++	+++	-	++	+++	++	+	-
19	+	-	-	-	-	+++	-	++	-	-	++	-	-	-
20	-	-	-	-	+++	-	-	-	-	-	+++	-	+	-
21	+	+	+	++	+++	-	++	-	+	-	+++	++	-	-
22	-	+	+	+	+++	-	++	-	++	+	+++	++	-	++
23	+	++	+	++	-	++	-	++	-	+	++	-	-	-
24	+	-	-	-	+++	-	-	++	++	-	+++	-	-	++
25	+	-	-	-	+++	-	++FN	++	-	++	+++	+++	++	-
Total	22/25	12/25	12/25	11/25	19/25	6/25	9/25	9/25	9/25	10/25	25/25	10/25	5/25	5/25

* - : No lesions observed; +: mild; ++ : moderate; +++: severe. ** Amount of antigen: - : negative; + : low; ++ : moderate; +++ : abundant. PPLI: Periportal leucocytic infiltration; ICA: Intrasinusoidal cellular aggregates; HD: Hydropic degeneration; BP: Bronchopneumonia; BIP: Bronchointerstitial pneumonia; FN: Focal necrosis

Lungs: Bronchopneumonia (19 fetuses) or bronchointerstitial pneumonia (6 fetuses) were the most prominent changes in the fetuses examined. The lesions were composed mainly of intraluminal and bronchial infiltration of macrophages and some neutrophils. Occasional macrophages and neutrophils were present in the interalveolar septa with edema (Plate V, Figs 1, 2). These changes tended to have a multifocal distribution. In fetuses with severe bronchopneumonia, moderate to large amounts of fibrin, cellular debris, macrophages and neutrophils were observed in exudate in the bronchioles and alveoli. The changes were also evident in the mucosa of the bronchi or bronchioles of these fetuses. Smaller airways were often partially plugged by macrophages and cell debris. Vascular hyperemia and perivascular accumulations of macrophages and neutrophils were the other microscopic findings. In three fetuses, there was amniotic debris and liquid in the airways. In the airways of the other infected fetuses there was mild to moderate alveolar thickening with moderate numbers of macrophages and neutrophils. In the affected fetuses, frequently, a diffuse reaction involving many adjacent lobules or an entire lung lobe were noticed.

Table 2
Comparison of histopathological findings and immunohistochemical results in the other fetal organs

Fetus	Histopathologic lesions*					Immunohistochemistry**				
No.	B	BLN	T	SI	A	B	BLN	T	SI	A
2	+	-	-	++	+	+	-	-	++	-
3	-	-	-	-	+	-	-	-	-	+
4	-	-	-	+	-	-	-	-	-	-
5	-	++	-	-	++	-	++	-	-	+
6	+	++	-	++	++	-	-	-	++	++
10	++	-	-	+	-	-	-	-	+	-
12	-	-	-	++	+	-	-	-	++	-
15	++	++	+	+	++	-	-	-	-	++
18	+++	-	+	-	-	+++	++	-	-	-
19	++	-	-	-	+	++	+	-	-	-
22	++	+	-	+++	++	+	++	-	++	+
23	+++	-	-	++	+	-	++	-	++	-
25	-	-	-	+	-	-	-	-	+	-
Total	8/13	4/13	2/13	9/13	9/13	4/13	5/13	0/13	7/13	5/13

* - : No lesions observed; +: mild; ++ : moderate; +++: severe. ** Amount of antigen: - : negative; + : low; ++ : moderate; +++ : abundant. B: Brain; BLN: Bronchial lymph node; T: Thymus; SI: Small intestine; A: Abomasum

Spleen: The majority of the fetuses had no splenic lesions. Changes were somewhat consistent between affected fetuses; however, severity of lesions varied. Prominent findings among 9 of the infected fetuses were slight lymphoid depletion of the white pulp and mild mononuclear infiltration of the red pulp. These changes accompanied by diffuse, and multifocal reticuloendothelial hyperplasia and mild lymphoid hyperplasia circumscribing splenic vessels. Two fetuses had intra- and subcapsular mononuclear leukocyte infiltration. In two other fetuses, small irregular area of necrosis in the splenic red pulp were seen.

Kidney: Small focal interstitial mononuclear leukocyte accumulations were present in the renal cortex and corticomedullar junction of 9 infected fetuses. Glomerular capillary tufts were dilated with hyperaemia.

Heart: Inflammation of the infected heart (9 fetuses) was generally mild and was characterized by the perivascular and myofibrillar infiltration of mononuclear leukocytes accompanied by an influx of some neutrophils.

Brain: Granulomatous meningitis was observed in 7/8 affected fetuses. Lesions were mild to moderate (6 fetuses) and severe in two fetuses. Cellular infiltrates in the affected fetuses with meningitis were predominantly lymphocytes and macrophages. Neutrophils were present in small numbers. The cells were arranged around arterioles and venules in the affected areas. The choroid plexus, at sites that were inflamed, had macrophages and lymphocytes, and most were within blood vessel lumens.

The changes in the bronchial lymph nodes from affected fetuses consisted of diffuse lymphoid hyperplasia in the cortex. Medullary cords were thickened by medium and large lymphoid cells. Sinusoids were dilated and infiltrated by small groups of neutrophils. Infrequently, focal areas composed of epitheloid cells were noticed in the cortical regions. A few thymuses had mild interlobular edema. There were no prominent changes in other fetal thymuses. Histologic examination revealed changes in the small intestines. Changes in the abomasum and small intestines were characterized by infiltration of mild-to-moderate numbers of macrophages, lymphocytes and small numbers of neutrophils in the lamina propria occasionally extending to the submucosa. The intestinal serosa was thickened with oedema.

Immunoperoxidase staining

Results of the immunohistochemical and bacteriological analyses are shown in Tables 1 and 2. *Brucella* specific staining was seen in sections of all the lungs (25 fetuses) examined. The specific staining was also visualized in other tissues and organs of fetuses with lesser numbers.

The positive staining was detected mainly in macrophages, and neutrophils in the exudates as brown, finely granular intracytoplasmic staining. Macrophages were large, dense, and their cytoplasm was filled with heavily stained amorphous material (Plate III, Figs. 1, 2). Cellular debris present in the alveoli and bronchi had intense reaction. In general, cells in the inflammatory foci revealed more intense reaction than isolated macrophages and neutrophils which were negative or had weak cytoplasmic reaction. Immunostaining in the liver was located in the cytoplasm of isolated macrophages in the portal infiltrates and Kupffer cells. Similarly, the antigens were present within cytoplasm of some hepatocytes and biliary duct epithelial cells as well as isolated macrophages in the sinusoids and interstitium. A few fetal kidney contained occasional mononuclear cells with low numbers of organisms immunoreactive for *B. abortus*. In the splenic sinusoids and red pulp, areas of positive cytoplasmic staining appeared in individual or small groups of large cells morphologically consistent with histiocytes and macrophages (Plate IV, Fig. 3). Brucellar antigen was demonstrated immunohistochemically in few mononuclear leukocytes in other fetal tissues and organs (Tables 1 and 2).

Bacteriology

In the study, vaccine strain (*B. abortus* strain 19) supplied from Pendik Central Veterinary Control and Research Institute (Istanbul, Turkey) was used to differentiation of field strains. No vaccine strain was found within the isolates. Microbiologically, 22 *B. abortus* biotypes were isolated from the 25 fetuses. Eighteen of the isolates were biotype 1, and 4 of were biotype 3.

Discussion

In this study, *Brucella abortus* positive aborted bovine fetuses developed histopathologic changes similar to those in experimental and natural infections in cattle (Enright et al. 1984; Palmer et al. 1996; Perez et al. 1998), sheep (Gorham et al. 1986; Yazıcıoğlu 1997) and bison (Rhyan et al. 2001). A series of pathologic changes in bovine fetuses infected with *B. abortus* occur including pneumonia, serohaemorrhagic lesions in body cavities and muscles (López et al. 1984; Hong et al. 1991). However, other lesions such as abnormal abomasal content, fibrinous pleuritis, vasculitis and meningitis occur less frequently (Hong et al. 1991).

Bovine fetal pneumonia is the most common lesion and is considered to be diagnostic of *Brucellae* infection by many (Enright 1984; López et al. 1984; Meador et al. 1989; Pérez et al. 1998). In the present study, characteristic pulmonary lesions were bronchopneumonia or interstitial pneumonia which are the most common lesions of aborted fetuses infected with *B. abortus* (López et al. 1984; Meador et al. 1989; Pérez et al. 1998). Although occurrence of bronchial necrosis (López et al. 1984; Pérez et al. 1998) and multinucleated giant cells (Enright et al. 1984) in inflammatory infiltrates have been described in other reports, such lesions were not seen in the present study. In this study, in only four cases bronch and bronchiol lumens contained aspirated amniotic fluid and generally considered an important *Brucellae* infection route in affected fetuses (López et al. 1984).

Hepatitis is a recognized sequela of chronic brucellosis in humans (Aygen 1998) and other animals (Elzer et al. 1998). In the present work, lesions in the liver and spleen

typically were diffuse reticuloendothelial hypertrophy, periportal and sinusoidal infiltration of a mixture of lymphocytes with smaller numbers of macrophages. Histological changes described in this report are similar to those recognized in cattle (Enright et al. 1984; Pérez et al. 1998) and sheep (Yazıcıoğlu 1997).

As in the present study, mild-to-moderate lymphoid hyperplasia circumscribing splenic arteries and splenic focal necrosis have been described in bovine fetuses (Hong et al. 1991), yet was not observed in caprine (Meador et al. 1988) and ovine (Yazıcıoğlu 1997) fetuses. Minor lesions have been reported in lymphoid tissues (Enright et al. 1984; Meador et al 1986). These lesions were lymph node hyperplasia, and presence of multinucleated giant cells with minimal necrosis in the cortical regions (Enright et al. 1984) which is a finding that was not seen in the present study. Minor lesions have also been described in brain, heart, kidney, and small intestines of *B. abortus* infected bovine fetuses (Enright et al. 1984; Hong et al. 1991), compatible with present findings.

High specificity and sensitivity were reported by immunohistochemical staining for the detection of *B. abortus* antigens in infected cows reaching 94% and 82%, respectively (Alberts and Erasmus 1995; Pérez et al. 1998). The specific granular immunohistochemical staining to the anti-*B. abortus* serum was detected mainly in the macrophage cytoplasm, in some neutrophils and cellular debris. An intense antigenic reaction was mainly localized in the cells of the inflammatory foci. However, some isolated inflammatory cells reacted weakly or negative. Similarly, previous reports shown that organisms were located mainly in the cytoplasm of the macrophages in the inflammatory foci (Meador et al. 1986; Pérez et al. 1998).

We found the low amount of *Brucella* antigen detectable in some sections of spleen, heart, kidney and liver from infected fetuses, even within areas of inflammation by use of immunohistochemical analysis which was consistent with other natural infections (Pérez et al. 1998). The number of *Brucella* organisms in these organs may have been less than the threshold of immunohistochemical detection. Alternatively, an already low number of bacteria may have been cleared rapidly from the organs following initiation of a latent inflammatory cascade that was seen histologically (Meador et al. 1986; Pérez et al. 1998).

In the present study, regional intrasplenic localization of cells with positive staining for *Brucella* antigen in affected fetuses were noticed. *In vitro* studies that used peritoneal macrophages have revealed that within 48 hours after inoculation, *B. melitensis* disrupts macrophage cell membranes, subsequently leading to a release of free bacteria in the external medium (Pizarro-Cerda et al. 1999). A reasonable explanation of the results seen histologically aborted fetuses reported here would be similar. This could ultimately result in an interregional splenic variation in bacterial burden. Although several sections of spleen were examined microscopically, it also was possible that areas containing bacteria were not included in all sections.

Meador et al. (1986) showed that bacterial culture is more sensitive than immunohistochemistry; in that study 9 of 14 caprine fetal tissues in which *B. abortus* isolated were positive by immunohistochemistry. Meador et al. (1986) reported that the minimum number of colony-forming units of *B. abortus* per gram tissue detected by the ABC immunostaining was approximately 106. However, in a more recent work a similar sensitivity of both immunohistochemistry and bacteriologic culture was shown (Pérez et al. 1998).

Brucella species and biotypes varies from country to country, even within the country (Villegas et al. 1999; Darwish and Benkirane 2001). *B. abortus* biovar 1 in Egypt, biovar 2 in Iran, biovar 3 in both Iran and Turkey were reported to be predominant biotypes for cattle (Refai 2002). There is limited study on the biotyping of *Brucella spp* from cattle abortion in Turkey. In this study, isolated *B. abortus* biotypes were identified as *B. abortus* biotype 1 (82%) and *B. abortus* biotype 3 (18%).

In the present study, three fetuses were positive by immunohistochemistry and negative by bacteriologic culture. The reasons possibly due to degenerated microorganisms, deficient isolation technique, or cross reaction of antibody with another antigen (López et al. 1984; Alberts 1995; Pérez et al. 1998). Although serologic detection of antibodies in cows was not possible it is regarded as the most sensitive method of the diagnosis of brucellosis because although dams may have antibodies to *Brucellae*, their fetuses may not develop infection, and concurrent placentitis can cause abortion without fetal infection (Palmer et al. 1996). Present findings support the findings of the previous reports that lungs are the most affected organs with the presence of bacteremia in the *B. abortus* infected fetus. It also reveals the use of immunohistochemistry as a complementary tool in suspected cases with negative bacteriologic culture, in cases when serology is not possible or material fixed in formaline.

Detekce antigenu *Brucella Abortus* z abortovaných bovinních fétů

Dvacetpět bovinních fétů přirozeně infikovaných *Brucella abortus* bylo morfologicky a imunohistochemicky vyhodnoceno společně s bakteriologickou kultivací. Histopatologické změny byly převážně bronchopneumonie, lymfoidní hyperplazie v játrech a slezině. Histopatologické změny v ostatních orgánech a tkáních odhalily hematogenní šíření infekce. Ve všech testovaných plicích byla zjištěna reakce imunitního systému na *Brucellu abortus* (25 fétů). Antigen však nebyl detekován v žádném z testovaných thymů. Intracelulární lokalizace antigenu byla zjištěna převážně v makrofázích, neutrofilech a hepatocytech. Navíc bylo z obsahu slezu a plic 22 fétů izolováno 18 kmenů *B. abortus*. Z těch bylo 18 biotypu 1 a zbývající 4 byly biotypu 3. Tyto nálezy jsou důkazem užitečnosti imunohistochemie v případech podezření, kde je bakteriologická kultivace negativní a v případech kdy je serologické vyšetření nemožné, či je materiál fixován ve formalínu.

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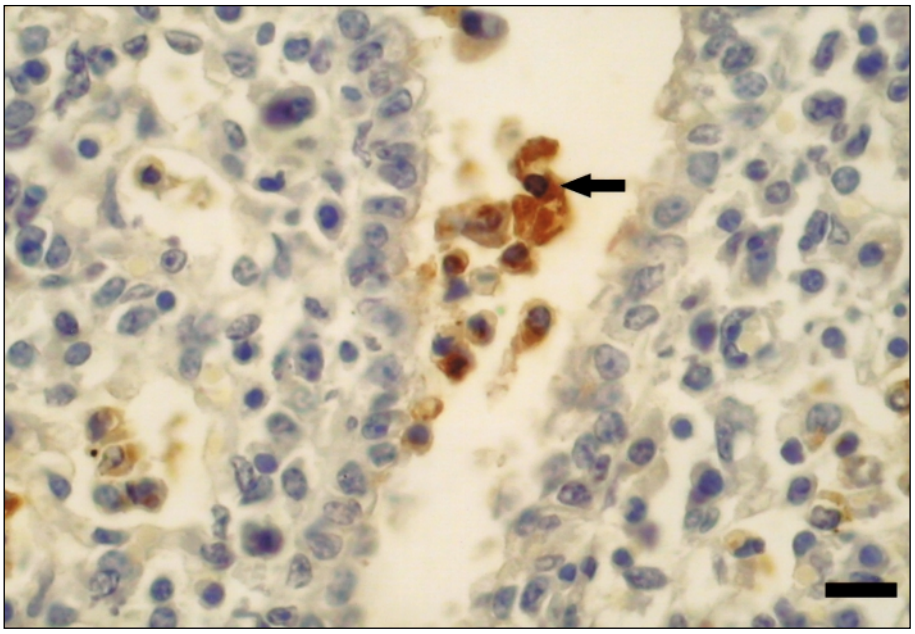

Fig. 1. Some macrophages located within the bronchiolar lumen show intense cytoplasmic immunoreactivity (arrow) to the anti-*Brucella abortus* polyclonal antibody. Fetus No. 8. ABC method. Bar = 22 µm.

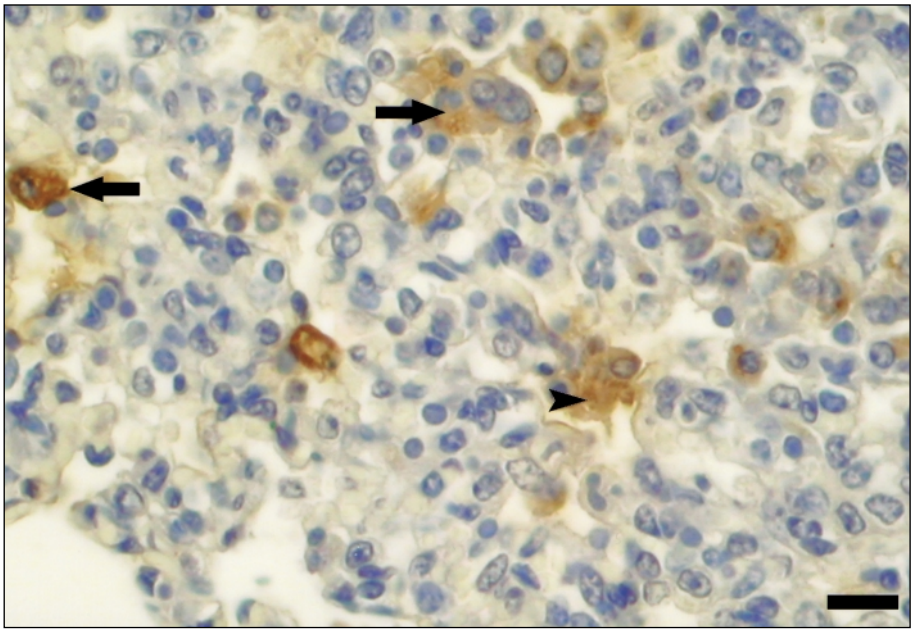

Fig. 2. Immunoreactivity to the anti-*Brucella abortus* polyclonal antibody in several macrophages (arrow head) and cellular debris (arrow) in a fetal lung. Fetus No. 3. ABC method. Bar = 22 µm.

Plate VI

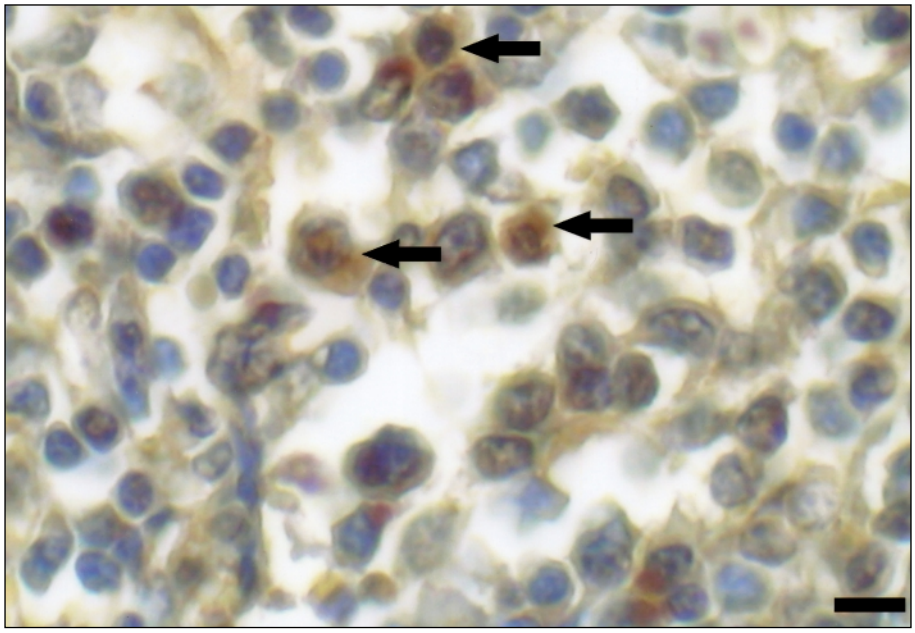

Fig. 3. Brucella-specific immunohistochemical cytoplasmic labeling within the cells of red pulp of a fetal spleen (arrows). Fetus No. 4. ABC method. Bar = 12 µm.