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APPLICATION OF IN SITU HYBRIDIZATION IN DIAGNOSIS OF BRUCELLOSIS IN TISSUES OF NATURALLY INFECTED SHEEP AND GOATS

(With 3 Tables and 7 Figures)

By

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تطبيق التهجين الموضعى فى تشخيص مرض البروسيلا فى أنسجة الأغنام والماعز المصابة طبيعياً بالمرض

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يعتبر مرض البروسيلا فى الأغنام والماعز من الأمراض الأكثر إنتشاراً فى جمهورية مصر العربية ويتم تشخيص المرض باستخدام الإختبارات السيرولوجية والبكتريولوجية. أجريت هذه الدراسة لتقييم حساسية ودقة طريقة التهجين الموضعى (التهجين الموضعى للحمض النووى داخل الخلية) مقارنة بالعزل البكتريولوجى للحيوانات. تم فحص عــدد مائــة حيوان (٥٠ أغنام ، ٥٠ ماعز) من مناطق مختلفة لها تاريخ إصابة بمرض البروسيلا باستخدام بعض الإختبارات السيرولوجية مثل إختبار الأنتجين المخمد المحمض واختبار الروزبنجال واختبار التلزن الأنبوبى البطئ واختبار المثبت المكمل. أظهرت نتائج هذه الدراسة عن وجود ١٩ حيوان إيجابى (١٠ أغنام ، ٩ ماعز) لاختبار واحد أو أكثر من الإختبارات السيرولوجية المذكورة. ومن هذه الحيوانات الإيجابية تم أخذ عينات من الطحال والعقد الليمفاوية للفحص البكتريولوجى والهستوباثولوجى واختبار التهجين الموضعى وكانت النتائج هى عزل ميكروب البروسيلا من ٧ حيوانات (٤ أغنام ، ٣ ماعز) وصنف الميكروب المعزول على أنه بروسيلا ميليتينسيس من النوع الثالث. أظهرت النتائج وجود ١٢ حيوان إيجابى باستخدام طريقة التهجين الموضعى. تم عمل مقارنة للنتائج البكتريولوجية ونتائج التهجين الموضعى أظهرت النتائج توافق الإختبارين فى الدقة بنسبة ١٠٠% وأن اختبار التهجين الموضعى ذو حساسية عالية أكثر من الزرع البكتريولوجى بالطرق التقليدية.

SUMMARRY

Brucellosis in sheep and goats are very common in Egypt and can be diagnosed by bacteriological and serological techniques. The present study was carried out for the assessment of sensitivity and specificity of in situ hybridization (ISH). One hundred animals (50 sheep and 50 goats) from an area with history of brucella infection were included and examined serologically by Buffered acidified plate test (BAPAT) Rose Bengal plate test (RBPT), Serum agglutination test (SAT), rivanol test and complement fixation test (CFT). Out of them 19 animals were reactors serologically by one or more test (10 from sheep and 9 from goats). From 19 reactors animals, proper samples from spleen and lymph nodes were collected for bacteriological, histopathological examination and ISH techniques. The results obtained from isolation trails, 7 brucella isolets were isolated (4 from sheep and 3 from goats). The isolated straines typed as Brucella melitensis biovar 3. The results obtained by ISH on paraffin embedded section of lymph nodes using a digoxigenin labeled DNA fragment located at gene encoding at 31 KDa Brucella abortus protein, revealed 12 cases out of 19 reactors animals were positive lymph nodes. The sensitivity of ISH techniques was higher than that of microbial culture where as the specificity of ISH was 100% when compared with the conventional microbial culture.

Key words: *Hybridization, Brucellosis, Sheep, Goats.*

INTRODUCTION

Brucellosis is one of the most important zoonotic diseases in the world. Ovine and caprine brucellosis still constitutes a serious problem in Egypt due to economic losses and their role in the transmission of infection to cattle and man. Control of brucellosis depends mainly upon the use of efficient serodiagnostic tests. However, no single test is capable to identifying all positive cases. Successful control of the disease needs an efficient diagnostic method. Complement fixation test (CFT) and Rose Bengal tests were selected for surveillance of ovine and caprine brucellosis (OIE 2004), but tests like buffer acidified plate agglutination (BAPA), rivanol test and mercapto ethanol test (ME) considered as confirmatory tools for serological diagnosis of brucellosis. Although the sensitivity of these diagnostic tests for the detection of specific antibodies against brucella species ranged from 65% to 95%, their specification can be low in areas where brucellosis is endemic,

mainly due to the high prevalence of antibodies in healthy population (Amann *et al*, 1995and Nicoletti, 1969). Moreover, other gram negative bacteria may cross-react with smooth brucella species and vaccinated animals can also give fals positive results in such test (Corbel *et al* 1984).

The gold standard that confirms the disease is the isolation of the bacterial agent (El Gibally *et al* 1977) and (Montasser *et al* 2001); subsequently the strain should be biotyped to identify its species and biovar (El-Gibally 1993). The biotyping of Brucella isolates can be performed by conventional bacteriological techniques. The isolation of etiological agent from clinical samples by conventional methods is not always possible, (Alton *et al* 1988). Thus, the development of new procedures for the detection and differentiation of brucellae is currently of great practical importance.

In situ hybridization (ISH) techniques allow specific nucleic acid sequences to be detected in morphologically preserved tissues sections. The techniques were described previously by (Gowans, *et al*, 1981) and (Burrel *et al*, 1984). At this time radioisotopes were the only labels available for nucleic acids and auto-radiography was the only means of detecting hybridized sequences. The fluorescent in situ hybridization (FISH) is a highly valuable tool for specific and rapid detection of pathogenic bacteria in clinical samples. In spite of FISH technique their use has been limited to research laboratories. This is probably due to radioactive probes, such as the safety measures required limited shelf life, the expense, the personal safety, the extensive time required for autoradiography and the isotope disposal problems (Brigati *et al* 1983). However, preparing nucleic acid probes with a suitable nonradioactive label removes the major obstacles which hinder the general application of ISH such as biotinylated probe and digoxigenin-labeled probe (Komminoth 1992).

The present investigation aimed to uses of in situ hybridization techniques in detection of brucella nucleic acid in the lymph nodes of sheep and goats which previously proved to be serologically positive. This was correlated with positive conventional bacteriological isolation and identification of Brucella melitensis in the target organs. In addition, the histopathological changes in these organs were noticed.

MATERIALS and METHODS

1. Serological examination:

A total of 100 serum samples (50 from sheep and 50 from goats), from an area with history of infection were individually collected and examined serologically by different serological tests. The applied serological tests namely Serum agglutination test (SAT), complement fixation test (CFT) and rivanol test (Alton *et al*, 1988), Rose Bengal plate test (RBPT) (Morgan *et al.*, 1969), and Buffered acidified plate test (BAPAT)-(Anon, 1984). The antigens of these tests obtained from Veterinary Vaccine and serum Research Institute, Abbassya, Cairo, Egypt. The Complement antigen was obtained from of the USDA's, National Vet. Services Lab.

2. Bacteriological examination:

Out of 100 serologically examined sera, 19 animals were reactors (10 from sheep and 9 from goats). From 19 reactors animals' proper samples from spleen, and lymph nodes (Supramammry, retropharyngeal and internal iliac lymph nodes) were collected under aseptic precautions and dispatched to the laboratory on ice as soon as possible. The direct cultural examination of spleen and lymph nodes samples were carried out under aseptic condition, inoculation into brucella agar media containing antibiotics and dyes. The plates were placed in incubator, which adjusted to 5-10 % carbon dioxide tension. Cultured plates were examined for brucella growth at 4th day and daily for 14 days, suspected colonies were further identified and re-subculture on brucella agar slopes.

The indirect culture, grinding of tissues samples were inoculated intra peritoneal into 2 guinea pigs. One of them was sacrificed after three weeks and the other one at six weeks from inoculation. Blood from guinea pigs was collected for serological examination at the time of scarification. Sacrificed animals were subjected to bacteriological examination for isolation of brucella organisms from the lymph nodes and internal organs. Identification of the brucella isolates was applied according to morphological characters, microscopical examination and reaction with positive sera.

The typing of brucella isolates was done according to CO_2 requirement, H_2S production, and growth in the presence of dyes (thionin and basic fuchsine), reaction with monospecific sera (A&M), and bacteriophage typing. All these procedures were done according to Alton *et al*, (1988).

3. In situ hybridization and histopathological examination:

Tissues specimens from sacrificed serological sheep and goats, (supramammary, and retropharyngeal lymph nodes) were collected and fixed in 10% neutral formalin. Tissues specimens dehydrated, cleared, embedded in paraffin, sectioned at 4-6 µm and stained with Harris haematoxylin and eosin stain according to Carleton *et al* (1967).

Another section from the same paraffin embedded lymph nodes were sliced at 5 µm thickness on coated glass slides with 2% 3-amino-propyltriethoxy saline in acetone "Sigma" to prevent falling of sections during the steps of hybridization. For the application of in situ hybridization on lymph nodes sections, specific probe for brucella was prepared. Genomic DNA was extracted from brucella culture according to methods described by Wilson (1990) and Vizcano, and Fernandez (1992). The probe used was a 223-bp (internal fragment located between bases of 789 and 1012 of the gene encoding a 31KDa Brucella abortus protein), labeled with digoxigenin (Roch). The preparation of lymph nodes section was followed according to Sambrook *et al,* (1989) and Saber *et al,* (1990). For prehybridization, slides were incubated for 2 hours at 42°C in 50% deionized formamide, 5XSSC, 5X Denhardts solution, 0.2 % SDS, 0.5 ug /ml calf thymus DNA.

Sections were then hybridized by incubation overnight at 42°C in hybridizing solution containing 100 ug /ml labeled olignucleotides (Rigby, *et al* 1977). Then the slides were incubated as follows:

Washing in 50% formomide 2X SSC for 30 min at 37°C

Washing in 50% formomide 1X SSC for 30 min at R.T

Washing in 1XSSC for 30 min at R.T. twice for each time

After hybridization and washing, section incubated for 1 hour at 50°C in blocking solution (0.1 M tris Hcl , pH 7.5 , 5 mM Mg cl_2 and 0.5 % triton X100) containing 3% BSA. Digoxegenin labeled probes were detected by a direct alkaline phosphatase system linked to anti-digoxigenin (Roche), by the methods described by Holtk, *et al,* (1995). Then non specifically bound complex was removed by three washes in Tris-saline-triton (0.1M Tris-Hcl pH7.5, 0.1M Nacl, 5mM Mg cl_2 and 0.05% tritonX100) for 15 min each. Alkaline phosphotase activity was then visualized by NBT and BCIP as colour reagent as described by the supplier (Bethesda Research Laboratory, Gaither burg, M D). Sections were then washed in water and mounted with gelatin/glycerol "Sigma" and covered by cover slips to be examined by light microscope. The positively brucella DNA was detected as bluish intra cytoplasmic dots.

RESULTS and DISCUSSION

Diagnosis of brucellosis in different countries of the world including Egypt depends mainly upon the application of different serological tests. In the present study different serodiagnostic tests including buffered acidified plate test (PABAT), Rose Bengal plate test serum agglutination test (SAT) rivanol test and complement fixation test (CFT), were employed for diagnosis of brucellosis. Out of 50 sheep serum samples examined, 18(36%) reactor for BAPAT, 12(24%) for Rose Bengal plate test, 14 (28%) for SAT, 13 (26%) for rivanol test and 15 (30%) for CFT in sheep respectively. While in goat the results revealed 15(30%) reactor for BAPAT, 11(22%) for Rose Bengal plate test, 9 (18%) for SAT, 9 (18%) for rivanol test and 10 (20%) for CFT in goats respectively as seen in table (1) and figure (1).

From the results obtained we found that, the high percentage of BAPAT, suggests the efficiency of these test as a screening test for detection of ovine brucellosis. (Stemshorn *et al.,* 1985), in comparison with other serological tests. While RBPT, gave a higher percentage than the SAT and this may attributed to, the SAT which may miss some infected animals especially those in the chronic stage of the disease (Morgan, 1969 and Wright, and Nielsen 1988).Moreover, the RBPT is more efficient in the detection of both early and chronic brucella infection (Morgan *et al.,*1969).

In this study, the result of different serodiagnostic tests applied on 50 goat serum samples revealed that the BAPAT showed the highest percentage of reactors followed by rose Bengal, CFT, SAT and Riv.T. These results emphasize the concept that the CFT achieve maximum balance between sensitivity and specificity (Stemshorn *et al.*, 1985)

In the present investigation we succeeded to isolate 7 brucella strains (4 from sheep and 3 from goats) as shown in Table (2&3). These samples were isolated from serologically positive animals. Biotyping of isolated strains indicated that all the isolated strains were Brucella melitensis biovar 3. The results of isolation coincided with those obtained by El-Gibally *et al* (1977) and Montasser and Mona Abdel wahab (2003). Brucella melitensis biovar 3 is mostly dominant strain recovered from different animal species in Egypt in last ten years. These findings reinsure the importance of using Rev 1 vaccine for vaccination of different animal species for proper eradication of brucellosis in Egypt.

In this study, we preferred to use digoxigenin–labeled probe for ISH due to its higher sensitivity and specificity as mention by Wang *et al.* (1993), and Komminoth (1992) who reported that digoxigenin

labeled probe considered a superior to radioactive probe and other non radioactive probe for ISH where it is stable for several months, provide an equal sensitivity in detection, turnaround time of procedure is short and quicker.

The results obtained in ISH on paraffin embedded section of lymph nodes using a digoxigenin labeled DNA fragment located at gene encoding at 31 KDa Brucella abortus protein, revealed 12 cases out of 19 reactors animals were positive lymph nodes. The positive sections is showed as a bluish intra-cytoplasmic dots in the lymphocyte (Figure 2 and 3) and in sinusoid (Figure 4) in comparison with control negative one (Figure 5).

On the other hand the bacteriological results by conventional techniques were compared by results of ISH. Out of 19 serological reactors cases were enrolled in this study, 10 from sheep and 9 from goats, 7/19 (36.84%) samples were positive diagnosed to brucella infection definitely by brucella culture. While the total detection rate of ISH for brucella infection in these samples was 12/19 (63.15%). The positive diagnosis rate of ISH in clinically diagnosed 7 brucella animals was 100% and in clinically suspected 12 animals was 5/12 (42%).

From the results obtained we found that, the specificity of ISH techniques when compared with the conventional microbial culture was 100% where as the sensitivity of ISH was higher than that of microbial culture. The false negative bacteriological results may be due to the low level of infection, massive contamination of samples, inhibition of some brucella species in the selective media, or by a viability loss before culturing and in all these circumstances DNA can still be detected by molecular DNA based techniques as ISH and PCR (Bail; *et al* 1992). These results come in harmony with that obtained by Blasco (1992), Gallien *et al* (1998) and Radwan and Ibrahium 2000). Similar findings were obtained by Ya *et al* (2001), when used PCR-ISH in diagnosis of mycobacterium tuberculosis. While Zerbi *et al* (2001), reported that the sensitivity and specificity of ISH in diagnosis of mycobacterium tuberculosis on formalin-fixed paraffin-embedded tissues samples were 100% and 95% respectively. The higher specificity of ISH was also recorded by Hopper *et al* (1989) and Fernandez *et al* (2000) when used ISH to detect brucella organism in tissues from experimentally inoculated mice with standard Brucella strains.

Pathological examination revealed oedema and enlargement of lymph nodes. The enlargements of the affected lymph nodes are due to reticuloendothelial hyperplasia together with infiltration of inflammatory

cells (Enany *et al* 1997). Microscopically, the lymph nodes showed lymphocytic depletion in germinal center of the lymphoid follicle Fig(6). Infiltration of blood vessels wall with round cells and histocytes was also detectd. Granuloma like structure in between lymphoid follicle consisted mainly from histocyte figure (7). These results agree with that obtained by Meador *et al* (1988), Montasser (1995) and Nashwa (2000).

CONCLUSION

The ISH was accurate and rapid technique when compared with conventional culturing methods of brucella organisms from tissues.

The ISH take 2-3 days for detection of DNA of brucella organism in paraffin embedded section in comparison with culture time which may extend to 14 days by direct isolation or 45 days by indirect isolation using experimental animals like guinea pig.

The higher specificity and sensitivity of ISH favours it's to use in detection of Brucella DNA in valuable registered animals by using biopsy from lymph nodes although it needs special procedures to collect samples and it is a little pet expensive.

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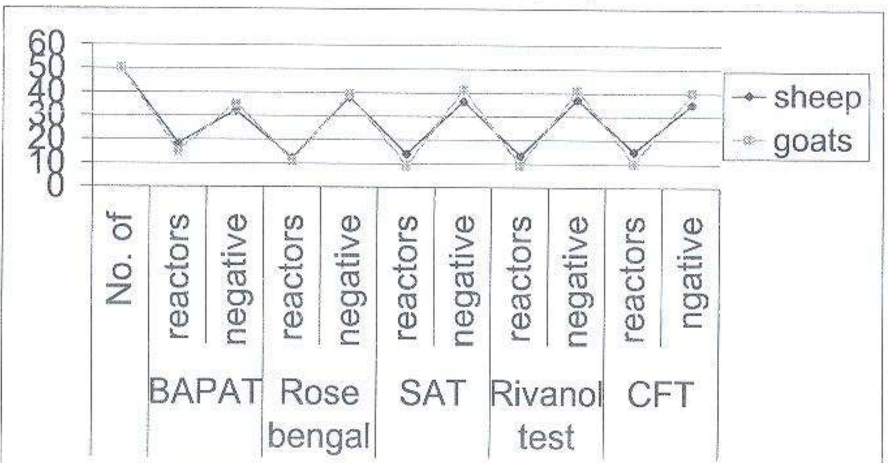
Table (1) Serological results of examined sheep and goats

		BAPAT		Rose bengal test		SAT		Rivanol test		CFT	
Animal spe	No. of anin	reactors	negative	reactors	negative	reactors	negative	reactors	negative	reactors	ngative
sheep	50	18	32	12	38	14	36	13	37	15	35
goats	50	15	35	11	39	9	41	9	41	10	40

Figure (1) illustrate the diagramed of serological results of examined sheep and goats



Table(2) : Results of bacteriological examination of spleen and lymph nodes compared with serological profile of 10 slaughered sheep

Sero. tests	Sero. Reaction	No. of animals	Bacteriological examination				Type of isolete
			Spleen	Ret. Ln.	Sup. Ln.	internal. Ln.	
BAPAT	Reactor	10	4	2	4	3	*Brucella melitensis biovar 3*
	Non reactor	0	0	0	0	0	
R.B.PT	Reactor	9	3	3	4	3	
	Non reactor	1	1	1	0	0	
SAT	Reactor	10	4	2	4	3	
	Non reactor	0	0	0	0	0	
Riv T	Reactor	9	3	3	4	3	
	Non reactor	1	0	1	0	0	
CFT	Reactor	10	3	2	4	2	
	Non reactor	0	0	0	0	0	

Table(3) : Results of bacteriological examination of spleen and lymph nodes compared with serological profile of 9 slaughered goats

Sero. tests	Sero. Reaction	No. of animals	Bacteriological examination				Type of isolete
			Spleen	Ret. Ln.	Sup. Ln.	internal. Ln.	
BAPAT	Reactor	9	3	2	3	2	*Brucella melitensis biovar 3*
	Non reactor	0	0	0	0	0	
R.B.PT	Reactor	7	3	3	3	3	
	Non reactor	2	0	0	1	0	
SAT	Reactor	10	3	1	3	2	
	Non reactor	0	0	0	0	0	
Riv T	Reactor	9	3	2	2	2	
	Non reactor	1	0	0	1	0	
CFT	Reactor	10	3	1	2	2	
	Non reactor	0	0	0	0	0	

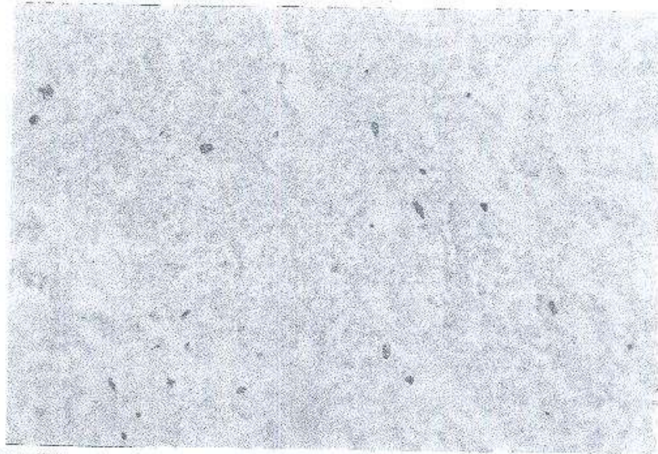


Figure (2) lymph nodes showing bluish cytoplasmic dots in the lymphocyte using ISH X400

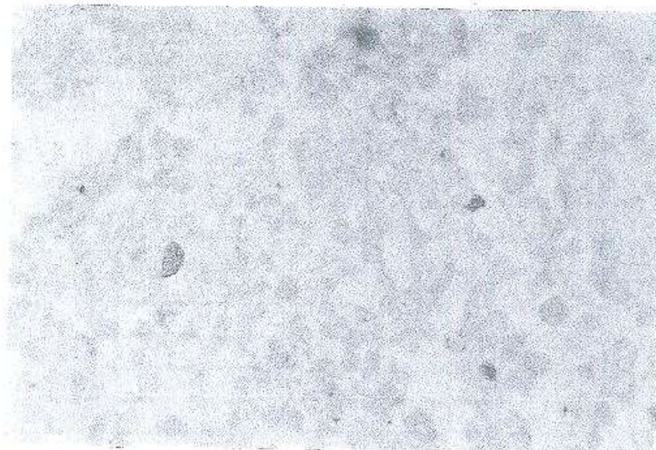


Figure (3) lymph nodes showing bluish cytoplasmic dots in the lymphocyte using ISH X1000

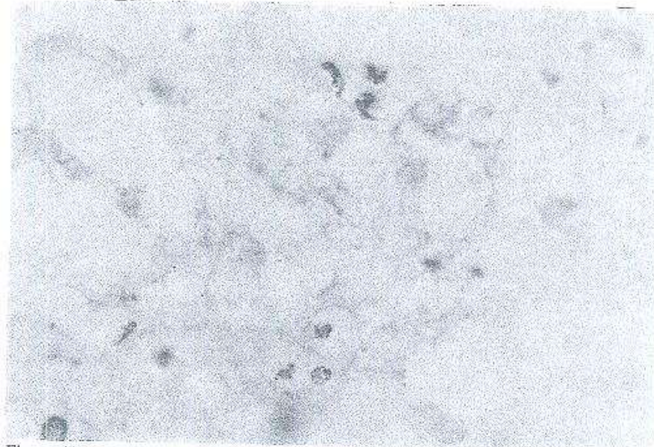


Figure (4) lymph nodes showing bluish cytoplasmic dots in the sinusoid using ISH X1000

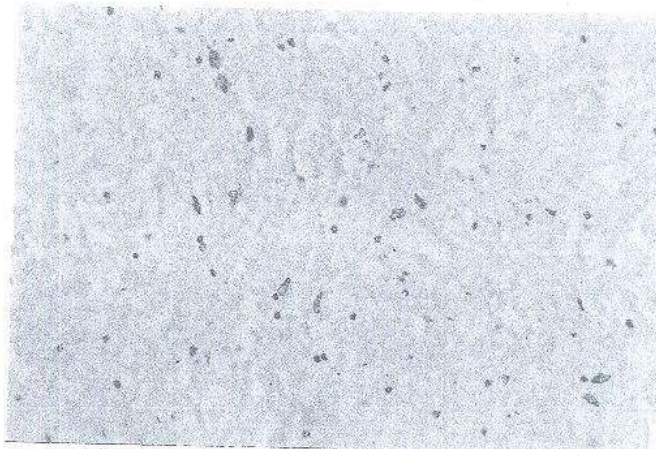


Figure (5) Lymph node as negative control showing no positive bluish dots in the lymphocyte using ISH X400

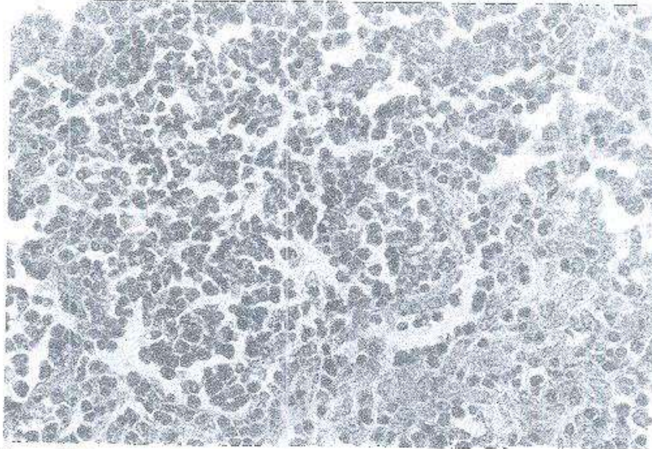


Figure (6) Lymph nodes showing lymphocytic depletion in germinal center of lymphoid follicle H&E X400

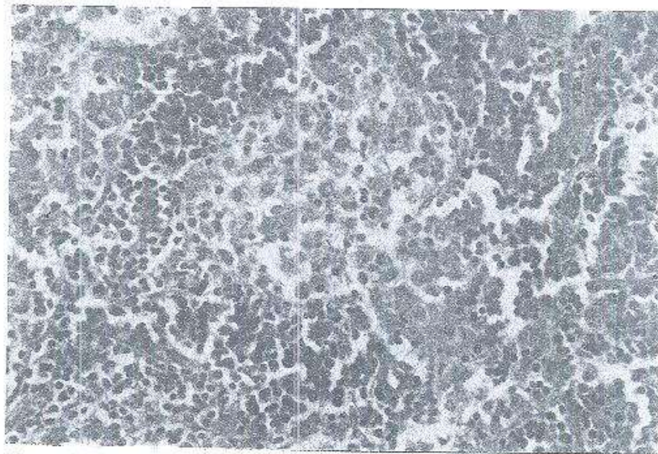


Figure (7) Lymph nodes showing histocyte aggregation forming granuloma like structure in between lymphoid follicle H&E X400