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AN ATTEMPT TO CONTROL THE MAJOR MANIFESTATIONS (ABORTION AND NERVOUS SYMPTOMS) ATTRIBUTED TO LISTERIA MONOCYTOGENES INFECTION IN SHEEP

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SUMMARY

A trial to control abortion and nervous symptoms in sheep was carried out through an experiment applied on 2 groups of pregnant ewes. First group included 6 ewes immunized S/C with 2ml suspension of local isolated field living L.monocytogenes (diluted $8X10^9$/ml) and challenged with 2 ml of the same strain of L.monocytogenes ($2x10^2$/ml). This group revealed gradual increase in the antibody titre till delivery using serum agglutination test (SAT) and ELISA. In addition, failure to reisolate L.monocytogenes in the rectal and vaginal swabs and no significant lesions characteristic for L.monocytogenes infection were detected pathologically. The second group (control group) consisted of 3 pregnant ewes, two of which challenged as the first group and the third ewe still alive and delivered normally and its lambs challenged as control group. The antibody titre of the 2 challenged pregnant ewes of this group (one died with nervous symptoms and the other aborted) was not present till challenge and increase somewhat after challenge. Moreover, L.monocytogenes was reisolated from rectal and vaginal swabs and from collected organs of dead ewe and from placenta of aborted one and from foeti. Histopathologically, the dead ewe revealed focal gliaosis, necrosed neurons, neurophagia, endothelial cell hyperplasia and perivascular infiltration by few number of lymphocytes and glia cells in the brain. Necrotic hepatocytes in the liver, follicular depletion in the spleen, exocytosis and focal areas of necrobiotic changes in the uterine epithelium and necrosis in the tips of the villi of the placenta. The foeti of dead and aborted ewes showed foci of coagulative necrosis, hemolysed R.B.Cs and hemosidrosis in the liver.

In this experimental work, a recommendation of using live preparation of *L.monocytogenes* vac-

cine has afforded a hope in control of listeriosis in pregnant ewes. There is a significant increase in antibody response in the serum of immunized dams and their lambs suggested the role of dam vaccination in protection of neonate against listeriosis.

INTRODUCTION

Listeria monocytogenes (*L.monocytogenes*) is an important pathogen of human beings and animals (Farber and Peterkin, 1991). It is widely distributed in nature and has been isolated from a wide varity of healthy and diseased mammals and birds species as well as from soil, water, sewage, mud and silage (Hyslop, 1974 and Blenden, 1986).

The prevalence of listeriosis has been increased in sheep and man during recent years (Anon, 1983; Low and Renton, 1985; Mclauchlin, 1987 and Anon, 1989).

Listeriosis caused by *L.monocytogenes* is characterized in some livestock species by three distinct syndromes of meningoencephalitis, abortion or still birth and neonatal septicaemia (Blood et al., 1983 and West and Obwolow, 1987). The three forms never overlap in the same animal and very rarely encephalitis-abortion syndrome can be reported. When outbreak occurs, all the animals affected manifest only one of these forms (Jubb et al., 1993 and Coetzer et al., 1994).

Meningoencephalitis most commonly occurs in ruminants and mainly characterized by incoordination, tilting of the head and the neck is pulled to one side. The animal moves in round and round in circles and called circling disease (Jubb et al., 1985). The brain showed oedema and small accumulation of lymphocytes and macrophages and sometimes a few neutrophils around or in close proximity to blood vessels of the brain. (Coetzer et al., 1994).

The aborted dams infected with *L.monocytogenes*, characterized by septiceamia and endometritis. The placental lesions included severe diffuse necrotizing and suppurative inflammations of both the cotyledons and the intercotyledonary areas. The aborted foeti mostly showed small yellowish pinpoint necrotic foci in the liver which appeared enlarged and pale or bronze friable liver (Donald et al., 2001).

The aim of the present work is to study the immunizing effect of live prepared vaccine in pregnant ewes and their off -springs against *L.monocytogenes* infection through measuring the antibody titers in serum and the bioassay methods and studying the pathological alterations occurring in the organs of unimmunized and immunized ewes and their off-springs.

MATERIAL AND METHODS

Animals:

1- A total of 33 (13 living and 20 dead) lambs

from different private farms, suffering from nervous symptoms (incoordination, unilateral paralysis of facial nerves, walking in circles, anorexia, salivation and followed by general paralysis and death) were used in this study. The gross pathological lesions were recorded.

2- Fifteen brucella free aborted ewes (10 living and 5 dead) and their foeti were involved in this study. Diagnosis of pregnancy in ewes and determination of fetal age were carried out by using ultrasound scanner 200 (Pie Medical-Mastricut-Netherland) equipped with3.5-5 MH2 linear array transabdominal transducer.

Experimental animals:

1-Three rabbits have been brought for Anton`s test.

2-Ten mice have been brought for pathogenicity tests.

3-Nine experimental pregnant ewes (2 months of pregnancy) have been brought and kept under observation for 2 weeks for studying the immunizing preparation.

Sampling:

Placenta, brain, uterus, liver and spleen were collected from dead animals showing nervous symptoms and from experimental animals (mice, lambs, dead aborted ewes and their foeti) for bacteriological and pathological studies. Rectal and vaginal swabs were obtained from the living lambs and aborted ewes for bacteriological study.

Isolation and identification of L.monocytogenes:

One gram of every specimen was macerated with one gram of sterilized sand. The emulsion was transferred to enrichment broth and incubated at 37°C for 24 hours. Subculturing on blood agar, listeria enrichment agar and tryptose agar were made and incubated at 37°C for 24-48 hours. "Beta" haemolysis was noticed on blood agar. Morphological examination by making smears of the suspected colony stained with Gram`s stain were examined microscopically. The isolates were examined for motility and Camp test with Staphylococcus aureus. The suspected *L.monocytogenes* isolates were identified biochemically according to Sneath et al. (1986).

Anton`s test: this test was used to study the biological characters of the isolated *L.monocytogenes*. it was done by instillating 2-3 drops of *L.monocytogenes* suspension into the conjuctiva of 3 rabbits (Anton, 1934).

Pathogenicity test: Intraperitoneal inoculation (I/P) of 5 mice with 0.25 ml of *L.monocytogenes* broth culture was done and 5 mice was remained as a control group (Emmerling et al., 1975).

Experimental immunization of the pregnant ewes:
Nine pregnant ewes (77-80 days of pregnancy) were classified into two groups. Three of them were used as control group and kept under hygienic measures in separate cage. The remained ewes

(6) were immunized subcutaneously (S/C) with prepared selected isolates of *L.monocytogenes* (2ml of diluted 8 X 10^9 /ml) using saponin as an adjuvant (Gudding et al., 1989). The experiment was done under complete strict hygienic and sanitation measures.

Serum samples were taken from ewes before immunization (0 day) and periodically every 15 days till delivery where the serum samples were collected periodically for 3 months (30 days intervals).

Rectal and vaginal samples were collected weekly during the time of experiment to reisolate *L.monocytogenes*. Rectal temperature was observed during the first week post immunization.

Experimental challenge the pregnant ewes:

Two weeks post immunization, ewes (6) and 2 ewes of control group (90-95 day pregnancy) were challenged S/C with 2ml of $2x10^2$ /ml of local isolated *L.monocytogenes* suspension after passage in mice to increase the virulence of the inoculated strains. (Gitter et al., 1986).

Rectal temperature was observed for one week post challenge. Rectal and vaginal swabs were collected periodically every week till parturition occurs.

Organs were collected from aborted and dead ewes and from their foeti for bacteriological and histopathological studies. Before parturition by 10 days, the remaining pregnant ewe was sacrificed and its organs (liver, spleen, uterus, placenta and brain) were collected for studying the histpathological changes due to immunization or challenge doses and also reisolation of L.monocytogenes.

Experimental challenged delivered lambs:

Seven lambs (3 months age) delivered from 4 immunized ewes and two lambs of the same age delivered from unimmunized ewe (control group) were challenged S/C with 1ml of $2x10^2$/ml L.monocytogenes after passage in mice according to Low and Donachie (1991). Rectal temperature was recorded for one week.

Hyperimmune serum was prepared in rabbits according to Low and Donachie (1991).

Serum samples were taken at time of birth (0day) and every month till reach 4 months of age.

The collected serum were used for measuring the titre of antibody using serum agglutination test (SAT) according to Gitter et al. (1986) and whole cell indirect ELISA according to Low and Donachie (1991). ELISA results were expressed as percentage optical density (OD) where:

$$\% \text{ OD} = \frac{(\text{mean OD test serum}) - \text{blank} \times 100}{(\text{mean OD hyperimmune serum}) - \text{blank}}$$

Histopathological examination :

Tissue specimens collected from dead and sacrificed experimental lambs, aborted ewes and their foeti and mice were fixed in 10% formal saline. After fixation, the tissue specimens were processed by conventional paraffin embedding technique, sectioned (3-4μm) and stained by hematoxylin and eosin (Harris, 1898) for routine histopathological examination.

RESULTS

Isolation and identification of L.monocytogenes:

Suspected colonies showed narrow zone of 'Beta' haemolysis on sheep blood agar and black colonies on listeria enrichment agar. They were Gram positive bacilli or coccobacilli and catalase positive. Tumbling type movement was seen in hanging drop preparation and characteristic umbrella pattern motility developed 3-4mm below the surface of semisolid motility media. *L.monocytogenes* was CAMP positive when cross-streaked with B-toxin producing S.aureus when subcultured on sheep blood agar. The suspected colonies produce acid with dextrose, L-rhaminose but not with manitol, D-xylose and sucrose. Fermentation of lactose was variable.

Anton`s test:

Isolated colonies produce purulent conjunctivitis within 24-48 hours, followed by keratitis in all rabbits.

Pathogenicity test in mice:

All inoculated mice died within 24-72 hours. *L.monocytogenes* were reisolated from liver, spleen and brain. Histopathologically, the liver showed moderate congestion, multiple sporadic necrotic cells forming focal areas of necrosis through the hepatic parenchyma and oedema in the portal area. Brain revealed congestion, central chromatolysis and neurophagia. The control group still alive.

Rate of isolation of L.monocytogenes in dead and living lambs, aborted ewes and their foeti:

Table (1) showed the occurrence of *L.monocytogenes* in lambs, 5 isolates (38.5%) were isolated from rectal swabs of 13 lambs showing nervous symptoms and 4 isolates (20%) were isolated and identified from parenchymatous organs of 20 lambs. Two isolates (20%) were identified as *L.monocytogenes* obtained from rectal and vaginal swabs and placenta of 10 aborted ewes and one isolate (20%) from organs of 5 aborted dead ewes. The foeti (16) revealed 3 isolates (18.8%) of L.monocytogenes.

Experimental challenge of immunized pregnant ewes:

One out of six pregnant ewes immunized with *L.monocytogenes* suspension aborted after one week of challenge without clinical symptoms. L.monocytogenes could not be reisolated from the collected organs, foeti and placenta.

Table (1): Rate of isolation of L.monocytogenes from lambs aborted ewes and their foeti.

Animals	Living		Dead	
	No.	%	No.	%
Lambs	5/13	38.5	4/20	20
Aborted ewes	2/10	20	1/5	20
Foeti	-	-	3/16	18.8

Table (2): Results of challenge of experimental immunized ewes and their lambs.

IMMUNIZED GROUP				**CONTROL GROUP**			
Aborted ewes		Dead lambs		aborted and dead ewes		Dead lambs	
No.	%	No.	%	No.	%	No.	%
1/6	16.6	0/7	0	2/3	66.6	2/2	100

Table (3): The serological responses of SAT and whole cell ELISA in serum of pregnant ewes before delivery.

Test	**Geometric mean log antibody titre in SAT**		**% of mean optical density (OD) in ELISA**	
Day	Imminized group	Control group	Immunized group	Control group
0	5	3.3	5	3.3
15	853	3.3	90.1	3.3
30	960	3.3	89.6	3.3
45	576	5	83.5	11.6
60	512	10	76.4	12.5
Before delivery	416	--	75.4	10

Table (4): The serological responses of SAT and whole cell ELISA in serum of delivered ewes

Test	**Geometric mean log antibody titre in SAT**		**% of mean optical density (OD) in ELISA**	
Day	Imminized group	Control group	Immunized group	Control group
0	384	10	75.2	5
30	304	10	74.4	5
60	272	10	72.6	5
90	192	10	75.2	5

Table (5): The serological responses of SAT and whole cell ELISA in serum of lambs delivered from experimentally immunized ewes.

Test	**Geometric mean log antibody titre in SAT**		**% of mean optical density (OD) in ELISA**	
Day	Imminized group	Control group	Immunized group	Control group
0	11.4	5	7.7	6
30	240	5	51.7	6
60	480	5	61.7	6
90	480	20	83.8	47.5
120	345	Dead	73.5	Dead

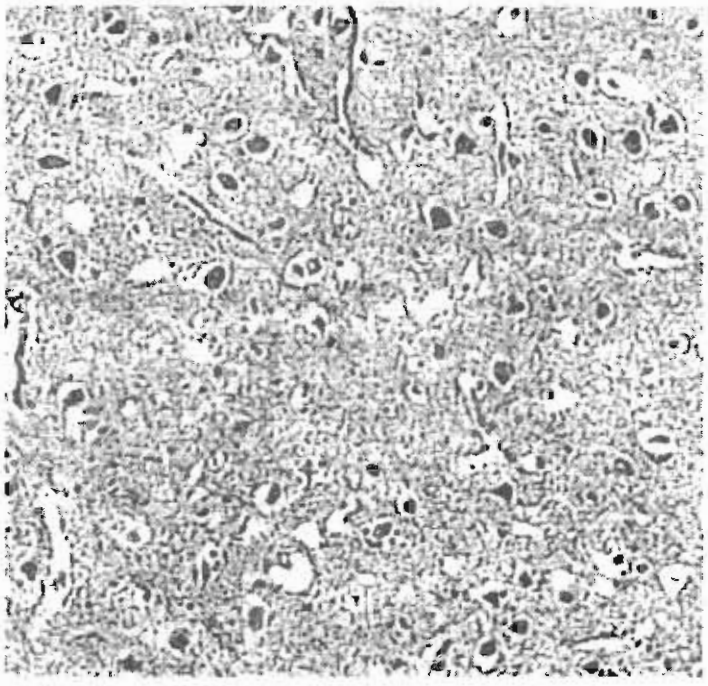

Fig. (1) Brain of challenged unimmunized dead ewe showing perivascular and pericellular oedema (X10).

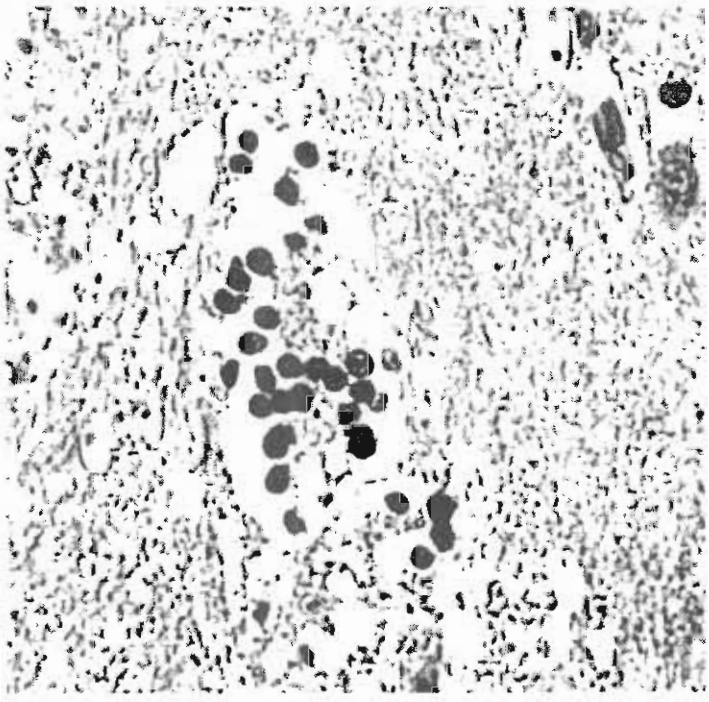

Fig. (2) Brain of challenged unimmunized dead ewe showing focal gliaosis (X40).

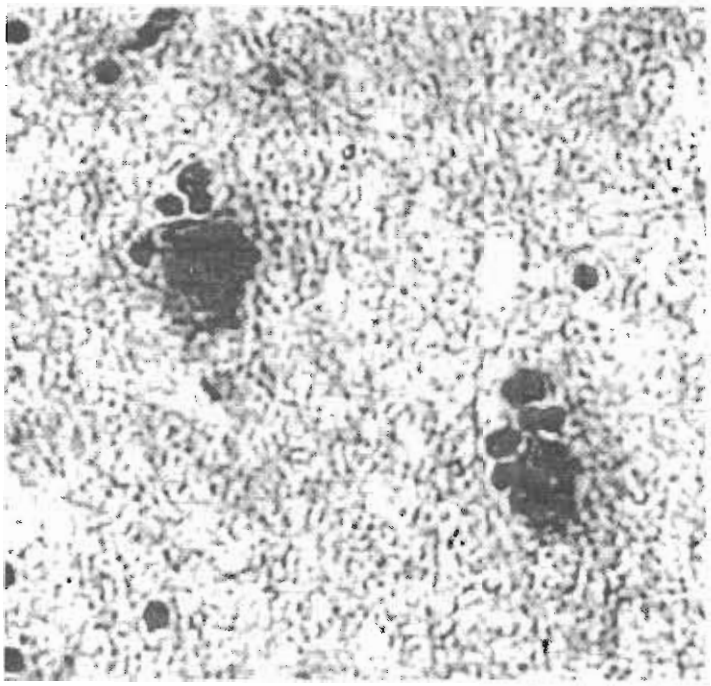

Fig. (3) Brain of challenged unimmunized dead ewe showing necrosed neurons and neurophagia (X40).

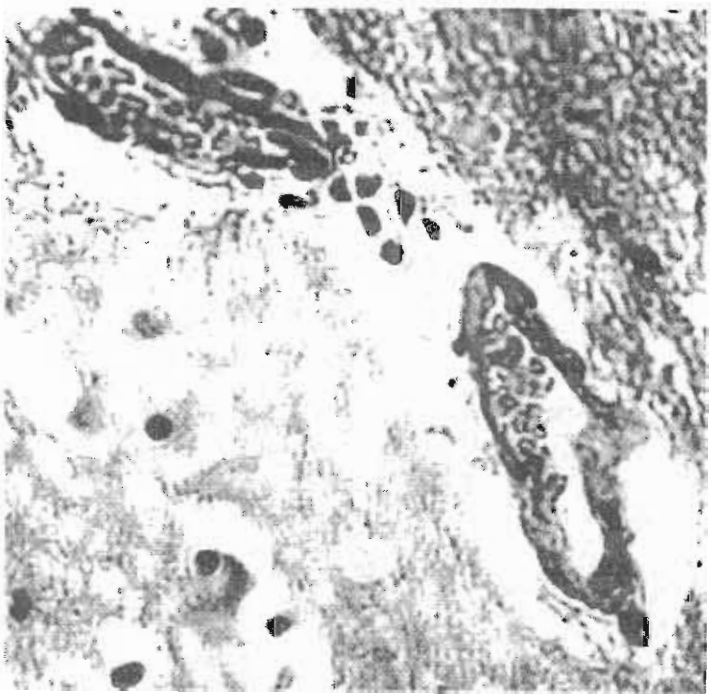

Fig. (4) Brain of challenged unimmunized dead ewe showing congested blood vessels, endothelial cells hyperplasia and perivascular infiltration by few number of lymphocytes and glia cells and oedema (X40).

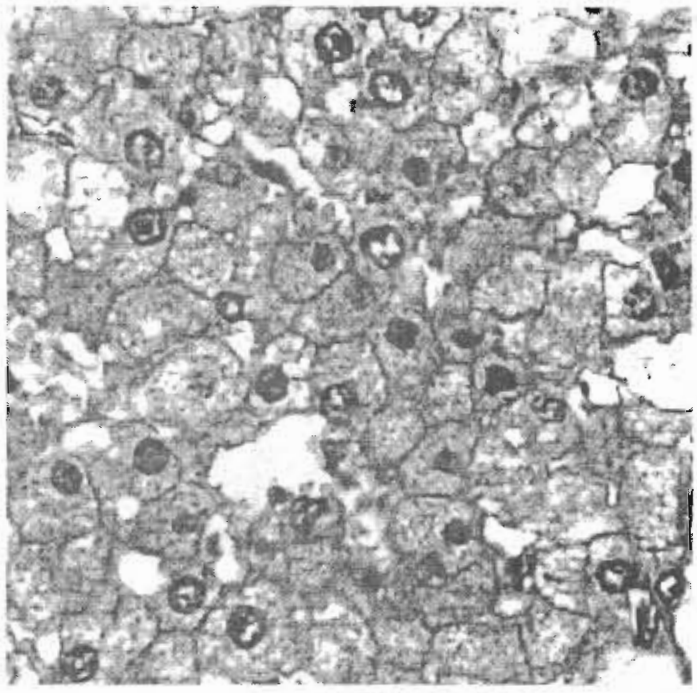

Fig. (5) Liver of challenged unimmunized dead ewe showing sporadic necrotized hepatocytes (X40).

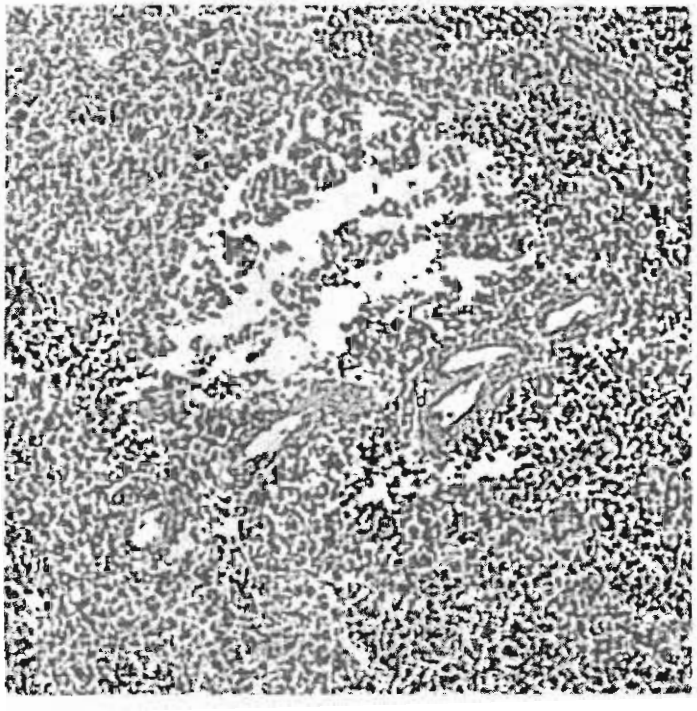

Fig. (6) Spleen of challenged unimmunized dead ewe showing follicular depletion (X10).

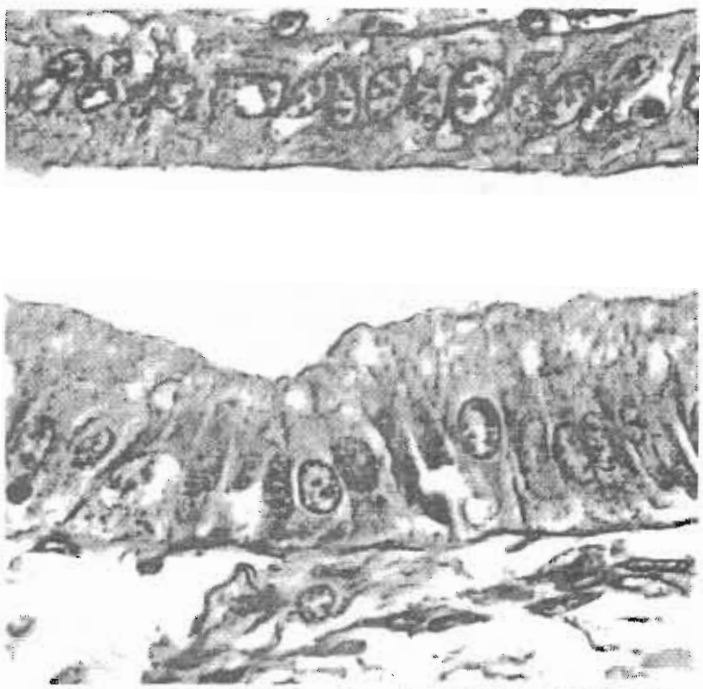

Fig. (7) Uterus of challenged unimmunized dead ewe showing exocytosis and focal areas of necrobiotic changes (X40).

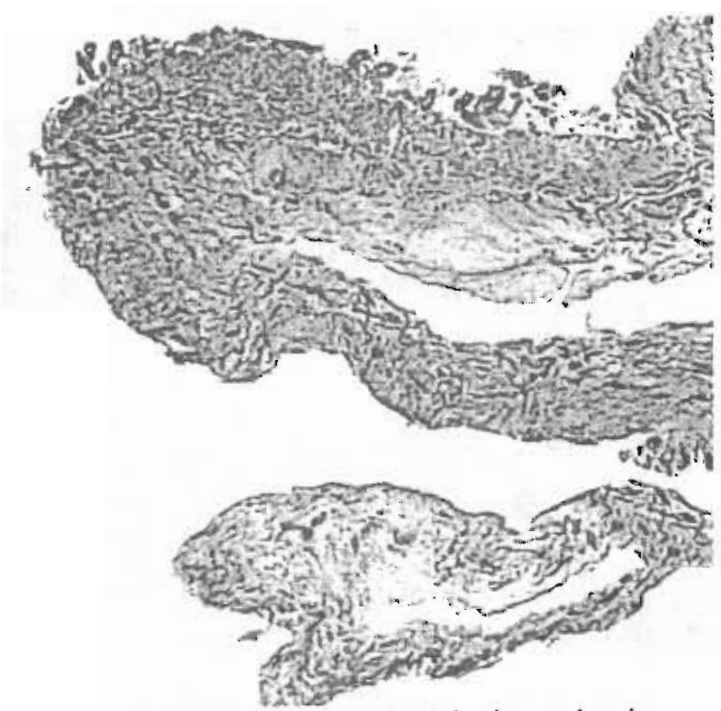

Fig. (8) Placenta of challenged unimmunized dead ewe showing necrosis of the tips of the villi (X10).

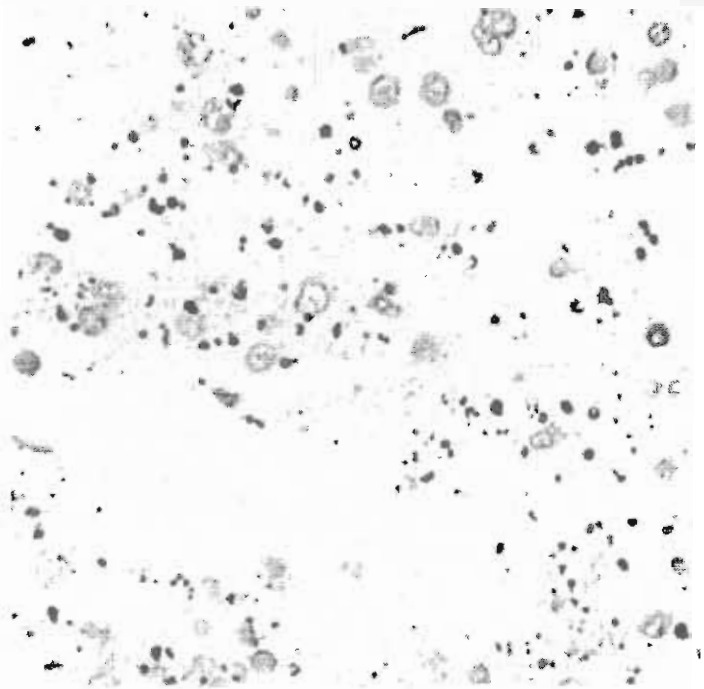

Fig. (9) Liver of aborted foetus showing coagulative necrosis, hemolysed RBCs and hemosidrosis (X40).

Fig. (10) Lamb experimentally infected with Listeria monocytogenes showing nervous symptoms (facial paralysis).

1280 and %OD was 92 after abortion. Pathologically, no significant lesions characteristic for L.monocytogenes infection were detected.

One of the immunized challenged ewes was sacrificed 10 days before delivery and reisolation of *L.monocytogenes* from its organs or its foeti failed. The geometric mean serum antibody titre was 640 and the %OD was 89 in ELISA. Pathological examination of its organs and foeti revealed no significant lesion macroscopically. Microscopically, the hepatocytes showed vacuolar degeneration, spleen revealed moderate congestion of the sinusoids of the red pulp and diffuse fibroblastic proliferation was seen in uterus. These histopathological findings are not characteristic for *L.monocytogenes* infection. L.monocytogenes was not reisolated from periodically collected rectal and vaginal swabs of the remained 4 immunized pregnant ewes and they delivered normally without clinical symptoms. The geometric mean antibody titres before and after delivery in SAT and the % OD of the antibody in ELISA were recorded in tables (3,4). It was revealed that the antibody titre was increased at 15 and 30 days post immunization and somewhat decreased after challenge and parturition.

With regard to bioassay of control group (two unimmunized ewes), one of them showed elevation in temperature (40-41.5°C) 3 days post challenge followed by nervous symptoms as circling and incoordination ending by paralysis and anorexia and death occur after 9 days (table 2). *L.monocytogenes* was reisolated from its organs including placenta, rectal and vaginal swabs and titre in SAT and the serological response in ELISA was increased after challenge (80,40 respectively). Post mortum examination of this ewe showed general septiceamia in all the carcass and its organs pathologically revealed:

Brain: Macroscopically, the brain showed severe congestion and peticheal hemorrhage and the meninges appeared congested and oedematous over cerebrum and cerebellum. Microscopically, it revealed perivascular and pericellular oedema characterized by dilated Virchow Robin spaces and hallow areas surrounding the neurons and glia cells (fig.1). Focal gliaosis (fig.2) and neuronal cell degeneration and necrosis associated with satelletosis and neurophagia (fig.3) were also seen. Moreover , the blood vessels showed congestion, endothelial cell hyperplasia and perivascular infiltration by few number of lymphocytes and glia cells (fig.4).

Liver: Macroscopically, the liver appeared enlarged and congested. Microscopically, sporadic necrotized hepatocytes (fig.5) which sometimes forming necrotic foci were seen through hepatic parenchyma and the portal areas showed mild infiltration of lymphocytes.

Spleen: No macroscopic lesion was observed in spleen. Microscopically, the spleen showed follicular depletion (fig.6) and hemosidrine granules.

Uterus: Macroscopically,the endometrium appeared swollen, congested and covered by mucinous clear or turbid exudates. Microscopically, the uterine epithelium showed partially desquamation,

exocytosis and focal areas of necrobiotic changes (fig.7). Diffuse oedema and congested blood vessels were noticed in the mucosa and submucosa and the uterine glands mostly appeared dilated and some of them showed destruction.

Placenta: Macroscopically, the placenta showed various degrees of oedema and sometimes grayish and white areas of necrosis were evident around the cotyledons. The placental villi appeared congested and covered by purulent exudates. Microscopically, it revealed diffuse oedema, severely congested blood vessels and necrosis of the tips of the villi (fig. 8) together with the infiltration of mononuclear round cells and few neutrophils.

The another challenged control unimmunized pregnant ewe showed elevation in temperature for three days and aborted after 10 days post challenge. *L.monocytogenes* was reisolated from placenta, rectal and vaginal swabs and from its foeti. The geometric antibody titre in SAT and the serological response in ELISA was increased after challenge (160, 40 respectively). Pathological examination of its placenta showed the same pathological picture observed in the placenta of the dead ewe.

Foeti: Foeti of dead and aborted ewes examined pathologically revealed general septiceamia and the liver was the most organ affected. Macroscopically, the liver appeared pale enlarged and showed tiny pin point yellow necrotic foci. Microscopically, multiple focal areas of coagulative necrosis, hemolysed R.B.CS. and hemosiderosis (fig.9) were evident through liver parenchyma. The fetal brain showed the same pathological pictures observed in the brain of the dead pregnant ewes to large extent.

The remaining control pregnant ewe was still alive without any clinical symptoms and delivered normally and its lambs were used as control group.

Experimental challenge of lambs:

Nine lambs of 2.5 months old challenged S/C with L.monocytogenes suspension (7 from immunized ewes and 2 from control ewe). Clinically, no symptoms appeared on the 7 lambs suckling from their immunized dams. But the control one showed elevation in temperature (41-41.5°C) with nervous symptoms which characterized by circling, incoordination, salivation, facial paralysis (fig.10) and ending with death after 10-12 days (table 2).

L.monocytogenes was reisolated from organs of the two dead lambs but could not be reisolated from rectal swabs of the other seven lambs. The geometric mean antibody titre in SAT and %OD in ELISA was described in table (5). It was noticed that the titre of antibody increased in the control group after challenge (120 in SAT and 47.5 in ELISA).

Pathological examination of liver, spleen and brain of dead lambs showed the same pathological picture of the dead pregnant ewe showed nervous symptoms.

DISCUSSION

In Egypt, sheep is considered as an important

source of animal protein. In this study L.monocytogenes were recovered from living lambs (38.5%) and 30% from dead ones, while it was 20% from both living and dead aborted ewes (table1), although the prevalence of *L.monocytogenes* is sporadic but it indicates occurrence of listeriosis in tropical and subtropical area as previously mentioned by Zakia et al. (1993) who recorded an outbreak of septiceamic listeriosis in sheep in Sudan. Anon (1983); low and Linklater (1985) and Anon (1989) reported that *L.monocytogenes* infection has increased in the recent years. On the the other hand Coetzer et al. (1994) reported that listeriosis is more common in countries and regions with a cold temperate climate than those with tropical or subtropical climate.

The biochemical activities of the isolates obtained from the present study were similar to that reported by Cruickshank et al. (1974) and Sneath et al. (1986).

Conjunctivitis and keratitis in all rabbits instillated with the isolated L.monocytogenes (Anton`s test) were similar to that reported by Sneath et al. (1986).

In pathogenicity test, death of all inoculated mice with septiceamic lesions and reisolation of L.monocytogenes proved the pathogenic nature of the isolates. This in agreement with Mackaness (1960); Lane and Unanue (1972); Abd El Wanees (1985) and Hassanein (1994).

Immunization of pregnant ewes with live vaccine revealed that one of the pregnant immunized ewe aborted after one week of challenge without clinical symptoms and pathological characterisitics for listeriosis infection. The failure of reisolation of *L.monocytogenes* from placenta and the fetal organs suggest that abortion occurs due to another causes rather than *L.monocytogenes*.

No characteristic pathological changes for listeriosis with failure of reisolation of L.monocytogenes were observed in the immunized challenged pregnant ewe sacrificed before delivery, meanwhile the normal delivery of the remaining immunized ewes and the high geometric serum antibody titre in SAT and %OD in ELISA revealed the efficacy of immunization and this was in agreement with Gudding et al. (1985) who recorded that no cases of listeriosis in vaccinated group. Coetzer et al. (1994) and Gudding et al. (1989) reported that experiment with live vaccine has given promising result in sheep abroad.

The geometric mean log of antibody titre and serological immune response in ELISA are useful in monitoring the development of antibodies against *L.monocytogenes* where antibody titre increased at 30 days post immunization and decrease slowly after challenge and after parturition (tables 3,4) The persistence antibody levels after challenge were confirmed by (Gitter et al., 1986). Lhopital et al. (1993) who reported that antibody levels almost stable for at least 4 months when injected ewes with a subclinical dose of viable *L.monocytogenes*. Low and Donachie (1991) recorded that the live *L.monocytogenes* are necessary for the production of immunity to listeric infection and the killed strains are in capable of stimulating any protection.

In control group, the clinical signs (abortion or nervous symptoms)and post mortem pictures reported in this study were in agreement with those reported by Vandegraff et al. (1981); Coetzer et al. (1994) and Donald et al. (2001).

The histopathological examination of these ewes clarified typical lesions of listeriosis in sheep which were described by Abd EL-Ghaffar and Abd El-Gwad (1997) and Donald et al. (2001) in the uterus and placenta and by Jubb et al. (1993) and Abd El-Ghaffar and Abd El-Gwad (1997) in the liver, as well as by Jubb et al. (1993) and Coetzer et al. (1994) in the brain. So, the necrotic changes observed in the placenta and uterus of challenged aborted and dead ewes play an important role in the occurring of abortion. Although, the typical clinical nervous symptoms of listeriosis were observed in the challenged pregnant ewes and lambs, histopathological lesions of the brain were not severe. This may attributed to the (S/C)route of experimental infection. In this concern, the natural route of infection for encephalitis is abrasions of the buccal or nasal mucosa or through conjunctiva. This suggestion is concurred with Radostits et al. (2000). Follicular depletion observed in the spleen of challenged unimmunized pregnant ewe give the attention to the effect of listeriosis on the lymphoid tissue and this need further investigation. The hemolysis of R.B.Cs. that prominently noticed in the liver of aborted foeti may be due to the power of hemolysin produced by L.monocytogenes which leading to destruction of red cells. This suggestion was supported by Jubb et al. (1993).

As regards to lambs delivered from immunized ewes,the geometric mean log titre of antibody of SAT and the immune response in ELISA in their serum increased gradually (from 11.4 to480 and 7.7 to 83.8 respectively)with significant decrease of antibody titre in serum of lambs suckling from unimmunized ewe, this indicates that there is maternal immunity required from milk. This suggestion is attributed with Bourry and Poutrel (1996) as they recorded that an IgG response in serum and milk increased significantly for 13 weeks in serum and for 3 weeks in milk of cows after challenge with *L.monocytogenes*. This findings need more study. The increase of antibody in control group of ewes and lambs after challenge in ELISA revealed that the serodiagnosis of listeria infection using ELISA as a rapid method is of significance. Low et al. (1992) reported that ELISA is a sensetive and reliable assay for detection of antibody against listeriolisin (LLO) antigen of *L.monocytogenes*.

The present trial has offered a promising result in sheep as vaccination of dams with live L.monocytogenes plays a significant role in protection of nconates and control of the major manifestations attributed to *L.monocytogenes* infection in sheep. In addition, histopathological examination is essential for the detection of the effect of immunization on the tissue and the detection of antibodies is useful for the serodiagnosis of listeriosis.

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