Bacteriological, Mycoplasmal, Virological and Pathological Studies on Mortalities in Imported Cattle

By
*Fatma, M. Darwish; **Soumaya, S.A. El-Shafii, ***Omayma A.S-hemies and ****Zeinab, R. Mohamed

*Pathology Department, **Bacteriology Department; ***Virology Department and ****Mycoplasma Department. Animal Health Research

**SUMMARY**

Mortalities in two imported Frisian cattle herds were investigated within two months after arrival in governmental quarantine at Suez governorate in 2006 and 2007.

The mortality rate was 8.34% in herd (1) 9.02% in herd (2) respectively. The diseased animals in the two herds showed respiratory manifestations, lacrimation, ptalism, mucopurulent nasal discharges, depression recumbency, anorexia and profuse watery diarrhoea.

The dead calves were necropized, gross lesions were reported and tissue specimens from internal organs were selected as well as nasal, ocular and rectal swabs were obtained from the diseased animals for histopathological, virological, bacteriological and mycoplasmal studies. Moreover, serum samples and blood samples on anticoagulant were collected from diseased and apparently healthy animals for virological and serological identification.

On virological studies, AGPT and commercial ELISA kits were rapid and accurate tests for detection of BVDV antigen. BVDV was isolated on MDBK cell line from buffy coats, nasal swabs and rectal swabs collected from diseased calves and from internal organs of dead ones, and was identified by IFAT using reference antisera. Also 64 serum samples collected from apparently healthy and diseased calves were tested by VNT for the detection of neutralizing antibodies against BVDV.

Moreover, the lungs, livers, kidneys of dead calves as well as nasal swabs of diseased ones yielded Mannhemia haemolytica on bacteriological investigation. The isolated strains were biotyped to biotype A (69 isolates) and biotype B (6 isolates). On virulence test, most M. haemolytica isolates were highly pathogenic for mice, where mortality occurs within 12-48 h. Meanwhile, the resistance of the isolates to most antimicrobial agents was high (resistant to ceftiofur, nalidixic acid, oxytetracycline, cephaloridine, gentamicin, cepalexin and donax and highly sensitive to norfloxacin, ampicillin and erythromycin).

Meanwhile Mycoplasma bovis were recovered from trachea, lungs, and livers of dead calves and nasal swabs of diseased ones.
Histopathological studies of dead calves depicted fibrinointerstitial bronchopneumonia, hepatocellular necrosis, necrobiotic changes of cardiac myocytes, immune complex glomerulonephritis (proliferative and membranous forms), lymphofollicular depletion of lymphoid cells in the spleen and mesenteric lymph nodes, necrotic changes of the ruminal and reticular mucosa, necrotizing enteritis with focal erosions and/or ulceration of the mucosa. The present investigation explored out that the principle cause of deaths was BVD viral infection which predispose and act synergistically with *M. haemolytica* and *M. bovis* through its immunosuppressive effect.

**Referred by**

Prof. Dr. Ahmed M. Ammar

Professor of Microbiology, Fac. Vet. Med., Zagazig University

Prof. Dr. Afaf El-khwas

Professor of Pathology, Animal Health Research Institute, Dokki

Prof. Dr. Nawal M. Ali

Professor of Virology, Animal Health Research Institute, Dokki

**INTRODUCTION**

Mortality in imported cattle is of great impact on beef industry since it has direct adverse effects on production (Chi *et al.*, 2002).

Most deaths in imported cattle occurred shortly after arrival and mainly in attribute to respiratory (shipping fever pneumonia, digestive or other disorders which are a consequence of interactions among stressors, host immunity, pathogenicity of infectious agents and environmental factors that alter the probability of pathogen exposure (Loneragan *et al.*, 2001 and Shehab *et al.*, 2001).

Beef industry suffers large economic losses from various infectious agents which hinder animal productivity represented by premature culling of infected animals, reduced slaughter value of the meat and reproductive losses (Chi *et al.*, 2002 and Hägglund *et al.*, 2007).

One of the most important infectious agents which infects cattle shortly after entering to the feedlot is BVD virus (Brodersen and Kel- ling, 1998). BVD virus has been associated with pathology of several physiologic systems including digestive, hematologic, immunologic, neurologic and reproductive systems (Grooms, 2004). Moreover, BVD virus is accorded a role in the causation of respiratory tract diseases and cause immune suppression as it acts synergistically with other pathogens results in more severe diseases.

BVD is a positive stranded RNA virus of the genus Pestivirus within family Flaviviridae (Ramirez *et al.*, 2001). Two biotypes,
non-cytopathic (ncp) and cytopathic (cp) are differentiated by their effect on susceptible cell cultures (Beknopet, et al., 2000).

The characteristic features of the Pestiviruses are the ability of the placental transmission of ncp-BVDV to infect the fetus at a particular stage of gestation resulting in persistently infected (PI) calves (Baker 1995). PI calves are considered the most important source of infection, excreting high amounts of virus continuously during their life via nasal discharge, saliva, semen, urine, feces, tears and milk (Fray et al., 2000).

Shipping fever pneumonia is precipitated by stress-inducing conditions such as shipping, viral infections, inhalation of diesel fumes and overcrowding (Frank, 1989) Mannheimia haemolytica resides in small numbers in the upper respiratory tracts of cattle (Shoo and Wisemens, 1990). Cells proliferate under stressful conditions and are aerolized in large numbers into lung alveoli, where they cause the disease (Frank and Briggs, 1992 and Gagea et al., 2006a).

Mycoplasma bovis is the most pathogenic mycoplasma species in countries free of contagious bovine pleuropneumonia. Naturally occurring infectious pneumonias of clinical significance usually have complex causes, after requiring the interaction of mycoplasma with two or more organisms and with predisposing environmental factors (Jubb et al., 1993). Mycoplasma is a proven cause of pneumonia; septic arthritis and keratoconjunctivities (Pfützner and Sachse, 1996 and Kirby and Nicholas, 1996).

M. Bovis is perced as an emerging cause of mortality in feedlot cattle associated with bronchopneumonia and arthritis and the relationship of this condition with BVDV infection as well as with bacterial infection with Pasteurella multocida. (Gagea et al., 2006a,b)

In Egypt, several studies have been implicated for the occurrence of deaths in imported cattle herds by Shehab et al. (2001) who related deaths to respiratory disorders due to mixed Mycoplasma bovis, Pasteurella multocida and streptococcal infection and Aly et al. (2003) who reported deaths due to outbreak of BVD viral infection either alone or mixed with other viruses in imported dairy herds quarantined at governmental quarantine of El-Beharia province.

The objective of the present study was to investigate the etiologic agents of mortalities in imported cattle herds from foreign

79
countries to Egypt on the basis of virological, bacteriological, mycoplasmal and pathological aspects. It is also a trial to identify the antibiogram of the isolates of bacterial pathogens.

MATERIAL AND METHODS

I. Animals:

Two Frisian cattle herds were imported from Romania to Egypt for slaughter purposes during November 2006 and March 2007 and quarantined at the governmental animal quarantine at El-Ein El-Sokhna (Suez province). The age of animals ranged from one to two years old.

a-Herd I: it consisted of 1354 male calves of which 113 calves were died within 10 days after arrival (the day of authors visit). The diseased animals in the herd suffered from respiratory manifestations (cough and dyspnea), ptyalism, lacrimation, and mucopurulent nasal discharge, incoordination, recumbency and anorexia.

b-Herd II: It was 2205 male calves of which 226 calves died within two months after arrival (the day of authors visit). The diseased animals displayed the same clinical signs observed in herd I, beside profuse watery diarrhoea, abdominal respiration and emaciation.

II. Samples:

- **Internal organs:** eleven dead calves were necropized in the two herds and tissue specimens from the internal organs were selected for virological, bacteriological, mycoplasmal and histopathological examination.

- **Swabs:** nasal, ocular and rectal swabs were obtained from 34 diseased calves for virological, bacteriological and mycoplasmal examination.

- **Serum and blood samples:** serum samples and blood samples on anticoagulant were collected from the same 34 diseased calves for serological diagnosis.

Tissue specimens were immediately immersed in 10% neutral buffered formaline solution meanwhile the other samples were collected in an ice box and transferred directly to the laboratory for diagnosis.

III. Virological examinations:

1. **Agar gel precipitation test (AGPT):** according to Hanel (1993)

   Samples (buffy coat, nasal swabs, ocular swabs, rectal swabs and internal organs) were tested against standard reference positive hyperimmune sera, the agar was
used in concentration of 1% in PBS and reaction was incubated for 24-48 h at 37°C in CO₂ incubator and then examined for the presence of precipitin line.

2– Institute pourquier ELISA kits:
These kits were used for detection of NSP₂-₃ and Eo of the bovine virus diarrhoea/mucosal disease virus (BVDV).

Assay procedure:
• Specific monoclonal antibodies directed to NSP₂-₃ and Eo are supplied and coated on the microplate wells
• The samples to be tested were incubated in the wells with a buffer containing polyclonal antibody directed specifically to BVDV, 50 µl of "sample incubation buffer" per well + 50 µl of sample to be tested per well and 50 µl of positive and negative control antigens (100 µl per well). The upper left two wells act as negative control and the next two wells act as positive control.
• The plate was incubated for 90 min. after covering with a lid. After 90 min. and after each step the plates were washed 3 times with washing buffer (if BVDV antigen were present in the analyzed samples a polyclonal antibody antigen, monoclonal antibody complex is formed).
• After washing, a peroxidase conjugated directed to polyclonal antibodies was incubated in wells for 30 min. 100 µl of diluted conjugate (1/10 in the wash solution) in presence of the BVDV antigen, NSP 2-3 or Eo in the samples, the conjugate binds on the plate.
• After washing the enzyme substrate (TMB) was added to the conjugate 100 µl/well and incubated at room temperature for 20 min, away from light, 100 µl of stopping solution was added per well, the plates were shaken gently to homogenate the colored solution. The recording of the plate was observed at optical density (OD) 450 nm wave length. The photometer must be first blanked on air.

Interpretation of the results:
Calculate for each sample the S/P ratio (in %)
S/P = OD of sample – OD of negative control / OD of positive control –OD of negative control x100

If sample S/P% < or = 25% are considered to be from animals that are not carrier to BVDV.
If sample S/P% is between 25%-30% are considered to be doubtful.
If sample S/P% = or > 30% are considered from animals carrier to the BVDV.
3- Cell culture:
A continuous cell line of Madian Darby Bovine Kidney (MDBK) was used for trials for isolation and propagation of BVDV from (buffy coat, nasal swabs, rectal swabs and internal organs) which was positive in ELISA test and also used in virus neutralization test, the cell proved to be free from non-cytopathic strains of BVDV. Monolayer cell line was grown in Eagles MEM supplemented with 10% fetal calf serum. The cell was obtained from virology department, Animal Health Research Institute (AHRI).

The collected samples after preparation were subjected for virus isolation via propagation on MDBK cell line according to the method described by Clark et al. (1984). Inoculated cells were incubated at 37°C and were examined daily for 5 days post incubation for three successive blind passages, CPE changes being to appear at the fourth passage.

4- Indirect immunofluorescent technique (Indirect IFAT):
The (IFAT) was used on the inoculated cell culture with cytopathic effect (CPE) to identify the (cp BVDV), it also was used on inoculated cell culture without CPE to detect (ncp BVDV). The indirect IFAT was carried out according to OIE standers (1992).

5- Enzyme conjugate:
Anti-bovine fluorescence isothiocyanate conjugate was supplied by Sigma immunochemicals used in IFAT.

6- Reference positive immune sera:
Standard reference positive bovine hyper-immune serum of BVDV was supplied by virology department AHRI

7- Virus neutralization test:
It was conducted for detection of specific BVD-V neutralizing antibodies in cattle serum samples according to OIE (2004).

IV- Bacteriological examination:
a- Cultural and biochemical identification:
A loopful of samples was sub-cultured on brain heart infusion agar supplemented with 5% defibrinated sheep blood and MacConkey agar. The plates were incubated at 36°C for 24 hs (Kodjo, et al. 1999). Bacterial identification was assessed by the observation of the colonial morphology, gram staining and biochemical identification including oxidase, catalase, urease testes, triple sugar iron agar, motility test and indol test (Atlas, 1997; Baily and Scott's, 1998 and Toply and Wilson, 1998).

b- Biotyping of isolated \textit{M.haemolytica}:
Biotyping of isolated *M. haemolytica* was applied depending on L-arabinose, trehalose, D-xylose, lactose and salicin fermentation tests (Biberstein *et al.*, 1990).

c- Virulence Studies of isolated *M. haemolytica*:

Eighty white mice were used in 3 groups to estimate the virulence of local strains isolated from different samples by inoculation S/C with 0.2 ml *M. haemolytica* 

(3x10^8) according to Refai (1990).

d- Antimicrobial susceptibility test:

The susceptibilities of isolates to antimicrobial agents were determined by using the disk diffusion method according to the NCCLS guidelines (2002). The antimicrobial disks used are Ampicillin, Amikacin, Ceftiofur, Cephaloridine, Cephalexin, Donax, Erythromycin, Gentamicin, Norfloxacin, Oxytetracyclin, Pencillin G Streptomycin and Nalidixic acid.

V- Mycoplasmal investigation:

1- Primary isolation of mycoplasma and Acholoplasma were done using heart infusion broth and agar as described by Sabry and Ahmed (1975).

2- Differentiation between mycoplasma and acholoplasma was done by using digitonin test according to Freurdt (1983).

3- Biochemical identification:

Glucose fermentation test: (Erno and Stiphovits, 1973):

The suspected cultures were inoculated into glucose medium and incubated at 37°C. The change of color from red to yellow or orange was considered positive.

VI- Pathological examination:

Representative tissue specimens from lungs, livers, hearts, kidneys, spleen, mesenteric lymph nodes, rumen, reticulum, small and large intestines were fixed in 10% neutral buffer formalin solution for at least 24 h. They were routinely processed by standard paraffin embedding technique, sectioned at 4 µ and stained with:

1– Hematoxylin and Eosin (Bancroft *et al.*, 1994)

2- Masson’s trichrome stain for detection of fibrous connective tissue according to Clayden (1971).

RESULTS

Virological studies:

Table (1) showed the results of comparison between AGPT and ELISA test, where only 33 samples (13.4%) were positive to BVDV by using AGPT while 104 samples (42.3%) were positive to BVDV by using ELISA test.

The isolation of BVDV from samples represented in Table (2), gave positive results in ELISA test (104 samples), where 43 samples (41.3%) gave signs of CPE, the cytopathic inducing viral agents were identified by IFAT, 37 out of 43 samples (35.5%) were identified as
Table (3) illustrates the results of BVDV specific antibodies in apparently healthy and diseased animals in serum samples by using VNT, 13 serum samples (43.3%) of apparently healthy animals were positive in a titer (1/4-1/16) while the diseased ones showed that (70.6%) serum samples were positive in a titer range (1/8-1/64).

**Bacteriological studies:**
Occurrence of *M. haemolytica* isolates in diseased and dead calves was illustrated in Table (4), where 75 *M. haemolytica* (41.2%) were isolated from samples of diseased and dead calves.

Sixty nine *M. haemolytica* were identified as biotype A and the rest (6) was identified as biotype T. (Table 5)

Virulence test of 75 *M. haemolytica* revealed that 67/69 mice were died within 12-24 hs (group 1) and the 5/6 died within 24-48hs and the control ones (5) still alive Table (6).

Antimicrobial susceptibility test revealed that *M. haemolytica* biotype A was highly sensitive to norfloxacin followed by ampicillin and erythromycin (65%, 50% and 40% respectively) and highly resistant to ceftiofur, nalidixic acid, gentamicine, cephaloridine, oxytetracycline and cephalexin (90%, 80%, 50%, 50%, and 45% respectively). Table (7)

*M. haemolytica* biotype T was highly sensitive to norfloxacin and erythromycin (75%, 50% respectively) and highly resistance to cephalixin, nalidixic acid, donax, and oxytetracycline (87.5%, 87.5%, 50% and 50% respectively). Table (8)

**Mycoplasmal studies:**
Primary isolation of mycoplasma yielded a total of 18 isolates from lungs of diseased calves and their associated lymph nodes with an incidence of 81.8%. The recovery rate of mycoplasma from pneumonic lungs of dead calves was 90.9% (10 isolates out of 11 samples) while 8 isolates were obtained from 11 associated mediastinal lymph nodes with recovery rate 72.7%.

The recovery rate of mycoplasma digitonin positive from positive specimens isolates were 9 (90%) from lungs and 7 (87.5%) from lymph nodes; 7 isolates (87.5%) from trachea; 5 isolates (83.3%) from liver; 4 isolates (80%) from spleen and 5 isolates (83.3%) from kidney (Table 9). In diseased calves, mycoplasma digitonin positive from positive nasal and ocular isolates was 12 (70.6%) and 12 (75%) respectively (Table 10).

There was one isolate typed as Acholoplasma from organs of dead calves, five isolates from nasal swabs and four isolates from ocular
swabs of diseased animals.

All mycoplasma isolates were proved to be *M. bovis*, where the isolates were positive to glucose fermentation, negative to arginine hydrolysis and positive to film and spot-formation.

**Gross pathology:**

The dead calves showed subcutaneous hemorrhages on most of the serosal surfaces, the hemorrhagic zones ranged from minute petechiae to ecchymotic hemorrhages. The thoracic cavity showed blood tinged straw colored exudates and in most cases, adhesion of the parital surface of the pleura to the inner surface of the thoracic cavity was evident. The pleura was extremely thickened and fibrotic, sometimes petechial hemorrhages were seen on its parital surfaces.

The lungs were bilaterally enlarged, edematous and congested with the appearance of multiple hemorrhagic foci ranged from punctuate to large ecchymatic hemorrhages. On cut section, whitish, grayish and/or reddish zones were observed (marbled appearance). Sometimes whitish creamy pus was oozed out from the pulmonary bronchi and bronchioles. Some parts of the pulmonary tissue exhibited irregular abnormal enlargements, on palpation; excessive amounts of air were recognized inside it. In one dead calf, both lungs exhibited variable sized abscesses in the pulmonary tissue. On cut section, creamy whitish pus oozed out. The mediastinal and tracheobronchial lymph nodes were enlarged, edematous and hemorrhagic.

The pericardium displayed minute petechial hemorrhages on its surface. The pericardial fat in some dead calves appeared pale, yellowish in color with gelatinous texture. The endocardium showed small focal areas of hemorrhages on its surface. Sometimes the myocardial muscles were soft and flappy in consistency.

The livers in most dead calves showed numerous whitish foci 2-5mm in diameter on its capsular surface. On cut section, it extended in the liver tissue shortly beneath the capsule.

The kidneys were pale in color with the presence of multiple whitish to grayish foci on the renal capsule. The ruminal mucosa was moderately congested and edematous. Small pale slightly elevated foci were observed on the mucosal surface.

The small and large intestines showed patchy congestion and hemorrhages on the serosal as well as on the mucosal surfaces. Multifocal sharply demarcated small erosions and/or ulcerations were noticed on the mucosa of the small intestine. The intestinal contents of both small and large intestines were watery and of greenish coloration. The mesen-
teric lymph nodes were enlarged, edematous, and hemorrhagic.

**Microscopic pathology:**

1- **Lung:**

The pulmonary blood vessels were engorged with blood with the appearance of focal hemorrhages in the pulmonary parenchyma. Some alveolar lumina were massively distended with faintly eosinophilic fibrillar network of fibrin which connecting in between alveoli through pores of Kohn in the alveolar walls.

The fibrinous exudate was studied with inflammatory cells in the form of histiocytes, lymphocytes and neutrophils (fibrinocellular pneumonia) (Fig. 2 A&B). Meanwhile, other alveoli showed thickened interalveolar septa due to proliferation of pneumocyte type II cells and infiltration with mononuclear inflammatory cells.

Some alveolar lumina showed presence of cellular exudates consisted of erythrocytes admixed with histiocytes, lymphocytes and neutrophils. (Fig. 3A). Moreover, other alveoli showed intralveolar aggregations of spindle-shaped or oat-shaped leukocytes which oriented in a swirly lamellations (Fig3 B).

In few instances, clumped exfoliated necrotic alveolar cells (strongly eosinophilic) were detected within the alveolar lumina admixed with inflammatory cells (Fig.4A). The pulmonary tissue showed presence of multifocal zones of coagulation necrosis which appreciated as circumscribed homogenous strongly eosinophilic area surrounded by histiocytes, lymphocytes, plasmacytes and neutrophils (Fig.4B). Sometimes, oat-shaped leukocytes were evident at the vicinity of the necrotic zone. Moreover, coccoid bacteria were heavily colonized at the periphery of some necrotic foci (Fig. 5A).

The interlobular septa were thickened by fibrous connective tissue proliferation that was infiltrated with lymphocytes, histiocytes and neutrophils.

The bronchi and bronchioles displayed necrobiotic changes of their lining epithelium as well as mononuclear cell infiltration in the mucosal layers with the presence of exudates in their lumina composed of exfoliated epithelium and cellular debris (Fig., 5B). Emphysema of some pulmonary alveoli was evident in many instances (Fig. 5B).

In one dead calf, numerous small to large abscesses were recognized in the pulmonary parenchyma and replaced considerable parts of the pulmonary tissue. Each abscess composed of a central caseated or calcified core surrounded by heavy aggregations of neutrophils, histiocytes and lymphocytes and bounded by fibrous connective tissue capsule. Thrombosis of some pulmonary blood vessels was observed associated with degenerative changes of
the tunica media (Fig. 6A). Moreover, vasculitis with marked degenerative and/or necrotic lesions of the endothelial layers was recognized in other blood vessels.

The pleura showed widely dilated blood vessels, focal hemorrhages and faintly eosinophilic fibrinous exudates intermingled with inflammatory cells composed of histiocytes, lymphocytes and neutrophils (Fig. 6B). Fibrous connective tissue proliferation was evident at some parts of the pleura.

2- Liver:

The hepatic tissue showed focal hemorrhages, disorganization of the hepatic cords, individualization of the hepatic cells and dilatation of the hepatic blood vessels and sinusoids. Necrobiotic changes of the hepatic cells emphasized by cloudy swelling and vacuolar degeneration of the hepatocytes and multifocal hepatocellular coagulation necrosis (Fig. 7A). The necrotic hepatocytes exhibited strongly homogenous eosinophilic cytoplasm with karyolysis of the nuclei. Bacterial bacilli were detected in the lumina of some hepatic blood vessels and hepatic sinusoids and/or in between degenerated or necrotic hepatocytes (Fig. 7B & 8A).

Increased fibrous connective tissue proliferation in the portal triads was evident in many instances and infiltrated with histiocytes, lymphocytes and neutrophils. Hyperplastic proliferation of the biliary epithelium with the formation of more than one bile ductules and focal injury of endothelial cells of some hepatic blood vessels was observed associated with mild to moderate vasculitis (Fig. 8B). Fibrous connective tissue proliferation in the Glisson’s capsule was detected in many instances.

3- Heart:

There were multifocal hemorrhages in between cardiac myocytes. The pericardial sac showed faintly eosinophilic edema fluid admixed with erythrocytes and mononuclear cells. Moreover, intermuscular and perivascular edema were also evident.

Some cardiac myocytes revealed vacuolar degeneration emphasized by intrasarcoplasmic vacuoles of variable sizes (Fig. 9A). Meanwhile, other cardiac myocytes exhibited necrotic lesions manifested by strongly eosinophilic sarcoplasm, loss of striation and karyolysis of the nuclei. In few instances, some cardiac myocytes appeared fragmented (Fig. 9B).

4- Kidney:

The renal blood vessels were engorged with blood. The renal interstitial tissue showed focal hemorrhages and mononuclear cell infiltration represented by histiocytes, lymphocytes and plasmacytes.

Some renal corpuscles showed hypercellularity of the glomerular
tufts due to proliferation and swelling of the endothelial and mesangial cells as well as due to neutrophilic and monocytic infiltration (proliferative glomerulonephritis) (Fig. 10A). The hypercellular glomerular tufts showed adhesion to the Bowman’s capsule and completely obliterated the Bowman’s space with marked obsolescence of the glomerular tufts.

Meanwhile, other glomerular tufts showed marked thickening of the glomerular basement membrane with proliferation of the endothelial and mesangial cells as well as inflammatory cell infiltration (membranous glomerulonephritis) (Fig. 10B). The tubular epithelium showed various pathological entities manifested by cloudy swelling and coagulation necrosis of the tubular epithelium with the presence of hyaline, epithelial or erythrocytic casts within the tubular lumina.

In one dead calf, chronic glomerulonephritis was recognized represented by extensive fibrous connective tissue proliferation in the renal interstitial tissue, periglomerular fibrosis and atrophied hypocellular glomerular tufts (Fig. 11A). Moreover, dystrophic calcification emphasized by deposition of basophilic structurless materials in the interstitial tissue, in some glomerular tufts and in some tubules was recognized (Fig. 11B).

5- Rumen and reticulum:

The mucosa of both rumen and reticulum exhibited group of cells showing degenerative and necrotic lesions in the stratum spinosum and stratum granulosum (Fig. 12). The cells were swollen with karyopyknosis or karyolysis of the nuclei. Mononuclear cell infiltration was seen in the lamina propria of both rumen and reticulum.

Sometimes the lamina epithelialis of both rumen and reticulum were heavily colonized by coccoid bacteria.

6- Small and large intestine:

The mucosal and submucosal blood vessels were engorged with blood. The intestinal mucosa showed coagulation necrosis of the surface enterocytes manifested by homogenous strongly eosinophilic cytoplasm with karyolysis of the nuclei (Fig. 13A). Sometimes, the brush borders of the necrotic villi were heavily colonized with coccoid bacteria (Fig. 13B). The surface enterocytes may be exfoliated into the intestinal lumina leaving microerosions. (Fig. 14A) or the entire mucosa may be completely exfoliated into the lumina and ulcerative enteritis was observed (Fig. 14B). In few instances, coccoid bacteria were admixed with necrotic debris of the exfoliated surface enterocytes. The cryptal enterocytes exhibited degeneration and necrotic changes and may be denuded into the cryptal lumina
The lamina propria showed edema with mild to heavy mononuclear and polymorphonuclear inflammatory cell infiltrations.

The mucosa of large intestine showed extensive infiltration with inflammatory cells in the form of lymphocytes, histiocytes and neutrophils. The intestinal crypts were either cystically dilated or showed hyperplasia of goblet cells (Fig. 15A).

The submucosal blood vessels exhibited fibrinoid necrosis emphasized by strongly eosinophilic homogenous fibrinous deposits in the tunica intima associated with mononuclear and polymorphonuclear leukocytic infiltration in the medial and adventitial layers (Fig. 15B). Edema around some of the submucosal blood vessels was evident.

The tunica muscularis were heavily infiltrated with mononuclear and polymorphonuclear leukocytes.

**7- Spleen:**

In all dead calves, marked depletion of lymphoid cells in the white pulp of the spleen with necrosis of some cells in the germinal centers was observed. (Fig. 16A). Moreover, necrosis of some lymphoid cells in the splenic parenchyma was evident in many instances. Golden brown hemosiderin pigment appeared to be dispersed in the splenic parenchyma. Splenic sinusoids were widely dilated and splenic trabeculae showed focal mononuclear cell infiltration.

**8- Mesenteric lymph nodes:**

Focal hemorrhages were observed in the parenchyma of the node and in some lymphoid follicles. The lymphoid follicles showed depletion of lymphoid cells with necrosis of some cells in the germinal centers leaving network of stromal cells.

Depletion of lymphoid cells was also evident in the cortical and paracortical zones as well as in the parenchyma of the lymph nodes. The lymphatic sinuses, subcapsular, peritrabecular and medullary sinuses were dilated and displayed presence of cellular exudates composed of erythrocytes admixed with histiocytes and lymphocytes (fig. 16B). Meanwhile other dilated lymphatic sinuses contained exaggerations of histiocytes within its lumina (sinus histiocytosis).

The mesenteric lymph nodes in one dead calf showed chronic lymphadenitis manifested by fibrous connective tissue proliferation in the parenchyma particularly around the lymphoid follicles (Fig. 17A). It was confirmed by using Masson’s trichrome stain (stained green) (Fig. 17B).
Table (1): Results of comparison between AGPT and ELISA test for detection of BVDV antigen in the diseased and dead animals.

<table>
<thead>
<tr>
<th>State of animals</th>
<th>Sample tested</th>
<th>No. of samples +ve by AGPT</th>
<th>No. of isolates +ve by ELISA test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>diseased</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffy coat</td>
<td>34</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td>Nasal swabs</td>
<td>34</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Ocular swabs</td>
<td>34</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rectal swabs</td>
<td>34</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td><strong>Dead</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trachea</td>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>11</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Spleen</td>
<td>11</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Mesentric L. N</td>
<td>11</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Kidney</td>
<td>11</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Heart</td>
<td>11</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Intestine</td>
<td>11</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Rumen</td>
<td>11</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Reticulum</td>
<td>11</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td><strong>Total No.</strong></td>
<td>246</td>
<td>33</td>
<td>104</td>
</tr>
<tr>
<td><strong>Overall %</strong></td>
<td></td>
<td>13.4</td>
<td>42.3</td>
</tr>
</tbody>
</table>

Table (2): Results of isolation and identification of BVDV from diseased and dead animals.

<table>
<thead>
<tr>
<th>State of animals</th>
<th>Sample tested</th>
<th>No. of samples with CPFT</th>
<th>Virus identification by IFAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diseased calves</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffy coat</td>
<td>22</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Nasal swabs</td>
<td>20</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Rectal swabs</td>
<td>8</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Liver</td>
<td>8</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Spleen</td>
<td>6</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Mesentric L. N</td>
<td>9</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Kidney</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Heart</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Intestine</td>
<td>8</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Rumen</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Reticulum</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>Dead calves</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>104</td>
<td>43</td>
<td>37</td>
</tr>
<tr>
<td><strong>Overall %</strong></td>
<td></td>
<td>41.3</td>
<td>35.5</td>
</tr>
</tbody>
</table>
Table (3): Result of BVDV specific antibodies in apparently healthy and diseased calves in serum samples by VNT.

<table>
<thead>
<tr>
<th>State of animals</th>
<th>No. of tested sera</th>
<th>No. of +ve</th>
<th>% of +ve</th>
<th>Average titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparently healthy</td>
<td>30</td>
<td>13</td>
<td>43.3</td>
<td>1/4-1/16</td>
</tr>
<tr>
<td>Diseased</td>
<td>34</td>
<td>24</td>
<td>70.6</td>
<td>1/8-1/64</td>
</tr>
<tr>
<td>Total No.</td>
<td>64</td>
<td>37</td>
<td>57.8</td>
<td></td>
</tr>
</tbody>
</table>

Table (4): Occurrence of *M. haemolytica* isolates in diseased and dead calves.

<table>
<thead>
<tr>
<th>State of animals</th>
<th>Type of samples</th>
<th>Total number of samples</th>
<th>Isolated <em>M. haemolytica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No.</td>
</tr>
<tr>
<td>Diseased calves (34)</td>
<td>Nasal swab</td>
<td>34</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Ocular swab</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Rectal swab</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>Dead calves (11)</td>
<td>lung</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>liver</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>spleen</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>kidney</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Gall bladder</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Lymph nodes</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>179</td>
<td>75</td>
<td>41.2</td>
</tr>
</tbody>
</table>

Table (5): Biotyping of isolated *M. haemolytica*

<table>
<thead>
<tr>
<th>No. of total <em>M. haemolytica</em> isolates</th>
<th>Biotype A</th>
<th>Biotype T</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>75</td>
<td>69</td>
<td>92</td>
</tr>
</tbody>
</table>
Table (6): Results of virulence test of isolated *M. haemolytica*

<table>
<thead>
<tr>
<th><em>M. haemolytica</em> biotype</th>
<th>Total No. of mice</th>
<th>Mortality</th>
<th>Time of death</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>Biotype A</td>
<td>69</td>
<td>67</td>
<td>97.1</td>
</tr>
<tr>
<td>Biotype T</td>
<td>6</td>
<td>5</td>
<td>83.3</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
<td>72</td>
<td>90</td>
</tr>
</tbody>
</table>

Table (7): Antimicrobial susceptibility tests of Biotype A (20 isolates as a representative isolates.

<table>
<thead>
<tr>
<th>Antimicrobial disk</th>
<th>Sensitive</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mild</td>
<td>moderate</td>
</tr>
<tr>
<td>Ampicillin 10µg</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Amikacin 30 µg</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Ceftiofur 30 µg</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Cephaloridine 30 µg</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Cephalexin 30 µg</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Donax 5 µg</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Erythromycin 15 µg</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Gentamicin 120 µg</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Norfloxacin 10 µg</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>oxytetracyclin 30 µg</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Pencillin G 10 units</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Streptomycin 10 µg</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Nalidixic acid 30 µg</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>
Table (8): Antimicrobial susceptibility tests of Biotype T (8 isolates).

<table>
<thead>
<tr>
<th>Antimicrobial disk</th>
<th>Sensitive</th>
<th></th>
<th></th>
<th></th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mild</td>
<td>moderate</td>
<td>strong</td>
<td></td>
<td>No.</td>
</tr>
<tr>
<td>Ampicillin 10µg</td>
<td>4</td>
<td>50</td>
<td>2</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin 30 µg</td>
<td>3</td>
<td>37.5</td>
<td>1</td>
<td>12.5</td>
<td>3</td>
</tr>
<tr>
<td>Ceftiofur 30 µg</td>
<td>2</td>
<td>25</td>
<td>1</td>
<td>12.5</td>
<td>0</td>
</tr>
<tr>
<td>Cephaloridine 30 µg</td>
<td>2</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Cephalexin 30 µg</td>
<td>1</td>
<td>12.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Donax 5 µg</td>
<td>2</td>
<td>25</td>
<td>1</td>
<td>12.5</td>
<td>0</td>
</tr>
<tr>
<td>Erythromycin 15 µg</td>
<td>1</td>
<td>12.5</td>
<td>1</td>
<td>12.5</td>
<td>4</td>
</tr>
<tr>
<td>Gentamicin 120 µg</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>Norfloxacin 10 µg</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>12.5</td>
<td>6</td>
</tr>
<tr>
<td>Oxytetracyclin 30 µg</td>
<td>1</td>
<td>12.5</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Pencillin G 10 units</td>
<td>1</td>
<td>12.5</td>
<td>1</td>
<td>12.5</td>
<td>3</td>
</tr>
<tr>
<td>Streptomycin 10 µg</td>
<td>1</td>
<td>12.5</td>
<td>3</td>
<td>37.5</td>
<td>3</td>
</tr>
<tr>
<td>Nalidixic acid 30 µg</td>
<td>1</td>
<td>12.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table (9): Recovery rate of mycoplasma and Acholoplasma from different organs collected from dead animals

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. of samples</th>
<th>Positive isolates</th>
<th>Mycoplasma</th>
<th>Acholoplasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Lung</td>
<td>11</td>
<td>10</td>
<td>90.9</td>
<td>9</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>11</td>
<td>8</td>
<td>72.7</td>
<td>7</td>
</tr>
<tr>
<td>Trachea</td>
<td>11</td>
<td>8</td>
<td>72.7</td>
<td>7</td>
</tr>
<tr>
<td>Liver</td>
<td>11</td>
<td>6</td>
<td>54.5</td>
<td>5</td>
</tr>
<tr>
<td>Spleen</td>
<td>11</td>
<td>5</td>
<td>45.5</td>
<td>4</td>
</tr>
<tr>
<td>kidney</td>
<td>11</td>
<td>6</td>
<td>54.5</td>
<td>5</td>
</tr>
</tbody>
</table>

Table (10): Results of recovery rate of mycoplasma from examined samples of living diseased animals and biochemical characterization of the obtained isolates.

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. of samples</th>
<th>Positive isolates</th>
<th>Mycoplasma</th>
<th>Acholoplasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Nasal swabs</td>
<td>34</td>
<td>17</td>
<td>50</td>
<td>12</td>
</tr>
<tr>
<td>Ocular swabs</td>
<td>34</td>
<td>16</td>
<td>47</td>
<td>12</td>
</tr>
</tbody>
</table>

* % was calculated according to number of positive samples.
Fig. (1): Normal MDBK non infected cells (A) and infected MDBK cells (notice specific intra-cytoplasmic fluorescent granules) (B).

Fig. (2 A & B): Lung of a dead calf illustrating fibrinous pneumonia, the alveolar lumina were massively distended with fibrinous exudate admixed with leukocytes (A & B. H & E x 400).
Fig. (3 A&B): Lung of a dead calf showing: A: Cellular exudate in the alveolar lumina. B: Spindle or oat-shaped leukocytes in the alveolar lumina which orientd in the form of a swirly lamellations. (A&B. H & E. A x 200 B x 400)

Fig. (4 A&B): Lung of a dead calf illustrating A: Strongly eosinophilic clumped exfoliated necrotic ma-
terial in the alveolar spaces admixed with inflammatory cells. B: Circumscribed zone of coagu-
lation necrosis (pulmonary sequestrum) bounded by aggregations of inflammatory cells (A&B. H&E A:x 100, B :x 200).
Fig. (5 A&B): Lung of a dead calf revealing: A: Heavy colonization of coccoid bacteria at the vicinity of the necrotic tissue. B: Degenerative and necrotic lesions of the bronchiolar epithelium with exfoliation into the lumina and emphysema of the adjacent alveoli. (A & B. H&E x 100)

Fig. (6 A & B): Lung of a dead calf showing: A: Thrombosis and degeneration of the tunica media of one of the pulmonary blood vessels. B: Fibrinouspleuritis; the pleura showed deposition of fibrinous exudate admixed with inflammatory cells (A&B. H &E A x 200, B x 100)
Fig. (7 A&B): Liver of a dead calf depicting: A: Focal hepatocellular coagulation necrosis. B: Widely dilated hepatic blood vessels with bacterial bacilli in their lumina (A & B. H & E x 400).

Fig. (8 A&B): Liver of a dead calf revealing: A: Bacterial bacilli in between necrotic hepatocytes. B: Intimal injury of the wall of one of the hepatic blood vessels. (A & B. H & E x 400).
Fig. (9 A&B): Heart of a dead calf illustrating: A: Vacuolar degeneration of the cardiac myocytes. B: Necrosis and fragmentation of the cardiac myocytes. (A & B, H&E x 400)

Fig. (10 A & B): Kidney of a dead calf illustrating: A: Hypercellularity of the glomerular tufts with adhesion to the Bowman’s capsule (proliferative glomerulonephritis). B: Membranous glomerulonephritis; the basement membrane of the glomerular tufts appeared thickened with mononuclear inflammatory cell infiltration. (A & B, H & E, A x 200, B x 400)
Fig. (11 A&B): Kidney of a dead calf showing: A: Chronic glomerulonephritis represented by periglomerular and interstitial fibrosis, hypocellular atrophied glomerular tufts and plasmacytic, lymphocytic infiltration of the renal tissue. B: Dystrophic calcification of the interstitial tissue and renal tubules. (A&B. H&E, A x 400, B x 200)

Fig. (12): Rumen of a dead calf showing degenerative and necrotic lesions in the cells of the stratum spinosum of the ruminal mucosa. (H&E x 400)
Fig. (13 A & B): Small intestine of a dead calf illustrating: A: Necrosis of both surface and cryptal enterocytes. B: Coagulation necrosis of the surface enterocytes and the brush border of the necrotic villi was heavily colonized with coccoid bacteria. (A&B H&E A x100, B x200)

Fig: (15 A & B): Large intestine of a dead calf revealing: A: Hyperplasia of goblet cells in the intestinal glands. B: Fibrinoid necrosis of one of the submucosal blood vessels associated with vasculitis and perivascular oedema. (A & B. H & E x 200)

Fig. (16 A & B): Spleen of a dead calf showing: A: Depletion of lymphoid cells in the white pulp of the spleen. B: Mesenteric lymph node of a dead calf illustrating dilated lymphatic sinuses that contained erythrocytes admixed with histiocytes and lymphocytes (A & B. H & E x 200)
DISCUSSION

In the present study mortalities in two imported Frisian cattle herds were documented within 60 days after arrival during 2006 and 2007 in governmental quarantine at El-Sokhna (Suez governorate). Most deaths were mainly related to respiratory diseases and digestive tract disorders. There are many factors that expected to compromise the immune function in animals after arrival encompassed change in animal health, management, environmental conditions, diets and commingling of animals as well as transportation stress. (Loneragan et al., 2001)

In Egypt, deaths in imported quarantined animals were previously recorded in fattening veal calves by Shehab et al., (2001) who attributed deaths to mycoplasmal pneumonia and Pasteurella multocida and Aly et al., (2003) who related deaths in 3 cattle herds to BVD viral infection either alone or mixed with other viruses.

The goal of this study is to isolate and identify the causative agents of these high mortalities and study their histopathological le-
sions in dead animals.

Amiridis et al. (2004) pointed out that BVB viral infection was responsible for severe deaths (22.85%) in 70 imported herds of Holstein heifers within six months after arrival.

The immunesuppressive effect of acute BVDV infections can enhance the clinical disease of other pathogens and play an important role in multiple infectious diseases (Potgieter, 1997). The immunesuppressive effect of BVDV mainly due to strong affinity of the virus for immuno-competent cells which may be destroyed or functionally impaired (Potgieter, 1995).

BVDV depresses immunoglobulin and interferon production, reduces response of lymphocytes to mitogens and impairs monocyte chemotaxis. This depression of cell mediated immunity is accompanied by reduced humeral immune response. (Jubb et al., 1993)

In the present study, AGPT was used as a simple and rapid method for BVDV or antigen detection in collected samples (buffy coat, nasal, ocular and rectal swabs and internal organs) which was reported by (Hanel, 1993; Hosny et al., 1996 and Nahed et al., 2004). Only 33 (13.4%) samples out of 246 samples were positive. Results of AGPT are low if compared with other tests due to the low sensitivity of the test or time of sample collection. Commercial Pourquier ELISA kits (antigen capture ELISA) gave rapid and accurate detection of BVDV antigen in the same original samples, where 104 (42.3%) out of 246 samples were positive.

An antigen capture ELISA was developed and proved its specificity, sensitivity and accuracy (Gottschalk et al. 1992). This coincidence with our view results of commercial ELISA kits for detection of BVDV in the field samples. Also, Donis (1995) reported that antigen detection ELISA for BVDV was useful mainly in confirming enteric, respiratory and reproductive diseases. Many authors described the importance of ELISA in testing program of animals to control BVDV (Sandvik et al., 1997 and Ferrari et al., 1999).

ELISA test was improved by the use of monoclonal antibodies which were specific to BVD viral proteins and we could detect most if not all BVDV strains (Sandvik, 1999). In our study we use kits with specific monoclonal antibodies directed to NSP2-3 (NSP-3 associated with lytic activity of the cytopathic strain) which increased the application of ELISA for detection of BVDV strains and offered sensitivity equivalent or higher than virus isolation, this also recorded by Ferrari et al. (1999); Cavirani et al. (2000) and OIE (2000). Also, Aymen (2002) proved that commercial ELISA kits are valuable in antigen detection.

MDBK cell line is considered the most common cell culture system for virus isolation and propagation (Allam, 2000)
In the present study, MDBK cells were used for three successive blind passages for samples positive by ELISA, out of 104 samples, 43 gave signs of CPE for BVDV on the cell. These samples were then subjected for identification by indirect immunofluorescent technique (IFAT) to detect non-cytopathic BVDV, where all gave negative results. Edwards (1990) and Brock (1991) reported that the conventional diagnosis of Pestivirus is based on direct detection of the virus in the clinical samples by using cell culture method followed by immunofluorescence. Also, Haines et al. (1992); Abd-El Rahim and Grunder (1996), reported that accurate diagnosis of BVDV infection depend upon isolating the virus from blood or nasal swabs or tissue samples from affected animals in diagnostic laboratory which agrees with our results.

The virus multiplication was detected by immunofluorescent technique that revealed diffuse or granular intracytoplasmic fluorescence in infected cells. The same results were reported by (Liess et al., 1974) who detected BVDV in organs of 11 from 14 dead cattle by (IFAT), also Munoz et al., (1996); Tsuboi and Imada (1999) and Zabal et al. (2000) used IFAT for identification of BVDV cell culture. The samples positive by ELISA kits and did not isolate or identify by IFAT may be due to the BVDV present in those negative samples FA complexes with antibodies rendered it non infectious for cell cultures (Palfiet et al., 1993). Improper handling or storage of the samples, instability BVDV, also ELISA can detect both BVDV and antigen. ELISA has advantage of speed handling large number and overcome low sensitivity other tests (Aymen, 2002). Serological examination of serum samples applied for detecting antibodies specific to BVDV is a useful tool for herd screening and BVDV prevalence and monitoring BVDV free herd status (Houe et al., 1995).

VNT was carried out for detection of specific neutralizing antibodies for BVDV in both diseased and apparently healthy animals. The results showed that 13 (43.3%) serum samples out of 30 apparently healthy animals were positive and 24 (70.6%) out 34 diseased animals were positive. Antibody tests were useful in assessing the status of animal groups as a part of disease control. VNT is the most common serologic test used as a reference method for BVDV tests (OIE, 2004).

M. haemolytica is a gram negative coccobacillus usually found as a commensal in the nasal passages of cattle (Abdullah et al., 1992). There are many predisposing factors such as, transportation, overcrowding and viral infection which may cause the M. haemolytica to shift from being commensal to pathogen (Confer et al., 1995). When a viral infection exists, the mucosal lining is degraded and the infection can move to the lower respiratory tract. Remaining defenses fail to resist infection because
of virulence factors of the bacteria.

*M. haemolytica* possesses various putative virulence determinants (Highlander, 2001) including a transferring receptors (Ogunnariwo and Schryver, 1990 and Yu et al., 1992); leukotoxin (Kaehler et al., 1980 and Davis et al., 1997) which are specific for ruminant transferin (Yu et al. 1992) and lymphoid cells (Berggen et al., 1981; Clinkeubeard et al., 1989; Davis and Lee, 2004 and Atapattu and Czuprynski, 2005). *M. haemolytica* also, produce toxins which inhibit the production of polymorphnuclear neutrophilic granulocyte (PMN), and chemotactic factors which kill bovines PMNs and macrophages (Morck et al., 1987 and Davis et al., 2001). However, capsular polysaccharides and lipopolysaccharides play a relative important role in the disease process. (Confer and Simons 1986; Adlam, 1989; Slocombe et al. 1990 and Davis et al. 1997).

In the present study, 75 *M. haemolytica* (42.3%) were isolated from dead and diseased calves. The high percentage of *M. haemolytica* may be attributed to more than one stress factors as shipping, viral infection (BVDV) and mycoplasma infection (Confer et al. 1995 and Highlander, 2001).

*M. haemolytica* were isolated from most specimens, this may be due to bacteremia (Baily and Scott’s, 1998).

Two biochemical types of *M. haemolytica* are recognized and are designated Biotype A and T, the letter stand for arabinose or trehalose fermentation (Adlam, 1989 and Muttlers et al., 1989).

Sixty nine (92%) of the isolated *M. hemolytica* were biotyped as biotype A and 6 *M. haemolytica* (8%) were biotyped as biotype T. These results revealed that *M. haemolytica* biotype A was the most frequently associated with shipping fever, a disease of beef cattle which characterized by fibrinous pleuropneumonia (Abdullah et al. 1992; Stevens and Czuprynski 1996; Reggie et al. 2001; Linda and Reggio 2002;Ewer et al. 2004 and Ilhan and Keles, 2007).

On virulence test, 90% of inoculated mice were died within 12-48h. About 97.1% of mice inoculated with biotype A were died within 12-24hs while about 83.3% of mice inoculated with biotype T were died within 24-48 hs. These results were nearly similar to that reported by Omnia (2006) and Odugbo et al. (2004) who reported the virulence of *M. haemolytica* biotype A on sheep.

On antimicrobial susceptibility tests, most of *M. haemolytica* were sensitive to norfloxacin, ampicillin and erythromycin. These results were agreed with Esaki et al. (2005) and Catry et al. (2007), they were resistant to ceftriaxone, nalidixic acid, gentamicine, cephaloridine, oxytetracycline, cephalaxin and donax. These results were nearly similar.
to that reported by Esaki et al. (2005) and Catry et al. (2005), and disagree with Mevius and Hartman (2000) and Berge et al. (2006), who recorded that M. haemolytica isolated from calves, sheep and goat were sensitive to ceftiofur, gentamicine and oxytetracycline. These results revealed that virulence of the isolated biotypes were high and treatment of M. haemolytica for respiratory tract disease is complicated by antimicrobial resistance. These may be attributed to the misused of antimicrobial drugs before laboratory diagnosis and applying of susceptibility tests, where the two herds were medicated by different antimicrobial agents before laboratory diagnosis.

In a survey conducted by the Office Internationals de Epizooties (OIE), M.bovis was a major component of calf pneumonia complex, with the isolation rates of 23.35% (Nicholas et al., 2000).

The situation of mycoplasma, a series of experiments designed by Houghton and Gourloy (1985), proved that there is synergism between pasteurella and Mycoplasma bovis in calf pneumonia. Also Abo El– Leil (1992) reported that in the extensive pleurisy, pasteurellosis and/or mycoplasmasis could be suspected. Moreover, Gagae et al. (2006a) reported the pathogens associated with mortality or severe morbidity for feedlot calves were BVDV, M.bovis and M.haemolytica.

In the present study, M.bovis was isolated from 9 (90%) from lungs and 7 (87.5%) from lymph nodes; 7 isolates (87.5%) from trachea; 5 isolates (83.3%) from liver; 4 isolates (80%) from spleen and 5 isolates (83.3%) from kidney. In diseased calves, M. bovis was isolated from nasal swabs 12 (70.6%) and ocular isolates 12 (75%).

These results were nearly similar to that reported by Gagea et al. (2006 a, b) who isolated M.bovis in a rate of 82% and is agree with Tenk et al. (2004) who screened 34 cattle for the presence of M.bovis, here it was isolated in a rate of 25.2%. The difference in the rate of isolation may be attributed to the number of examined samples.

At necropsy, the dead calves displayed fibrinointerstitial bronchopneumonia with pleuritis and adhesion of the parital surface of the pleura to the inner surface of the thoracic cavity. These morphological features may be related to severe toxemia incited by the infection with M.haemolytica as well as to mycoplasmal infection which brings about fibrinous bronchopneumonia and fibrinous pleuritis as mentioned by Gagea et al. (2006 a & b) and MacGavin and Zachary (2007).

Moreover, these results are consistently similar to Donkergoed et al. (1993) who isolated M.haemolytica from pneunmic dead calves in 73% and Gagea et al. (2006a,b) who recorded the common causes of pneumonia and death in imported beef feedlot calves within 2 months after arrival were M.haemolytica (27%) and M.bovis.
(82%) and M. arginini in (72%).

Histologically, the lung tissue exhibited inflammatory exudates within the alveolar lumina, either fibrinous, fibrinocellular or cellular which may be admixed with cellular debris. Jubb et al. (1993) suggested that, the production of profuse fibrinous exudate correlates with a fulminating pulmonary inflammation and usually develop in cattle that have been recently stressed by transportation. Moreover, the detection of fibrinocellular exudate (fibrin admixed with histiocytes, neutrophils and plasma cells) in other alveolar lumina as well as in the pleural sac was attributed by (MacGavin and Zachary, 2007) to increased permeability of the blood-air barrier, increased procoagulant activity and diminished profibrinolytic activity of the lung, furthermore, fibrin is chemotactic for leukocytes particularly neutrophils.

The tendency of leukocytic aggregates (elongated cells with spindle shape nuclei or oat shaped cells) in the alveolar lumina is considered one of the pathognomic lesions of pneumonic pastereullosis and attributed to the toxins from gram –ve bacteria as described by Jubb et al., 1993; Jones et al. 1997; MacGavin et al., 2001 and Blowey and Weaver, 2003. Meanwhile MacGavin and Zachary, 2007 suggested that oat-shaped leukocytes were degenerated neutrophils admixed with alveolar macrophages.

The detection of numerous coagulative pulmonary parenchymal necrotic zone bounded by leukocytic aggregates (pulmonary sequestra) is a true pathologic entity incited by the endotoxins as well as by leukotoxin of M. haemolytica that specifically destroy ruminants leukocytes (polymorphes, lymphocytes and histiocytes) at the site of infection and impairing immune response. (Bottone, 1998)

Another reason for extensive necrosis of lung tissue was related to the secretion of maximum amounts of cytokines by alveolar macrophages such as TNFα, interleukin I, and interleukin 8 with potent neutrophil chemotactic activities that contribute to injury and necrosis of the pulmonary tissue (Malazdrewish et al., 2001 and MacGavin and Zachary, 2007). Furthermore, vasculitis and thrombosis of the blood vessels incited by mycoplasmal infection may responsible for large pulmonary sequestra due to pulmonary infarction. (Shahriar et al., 2002 and Gagea et al., 2006a)

Moreover, bronchitis, bronchiolitis, pleuritis, thickened interlobular septa as well as thickened alveolar walls (interstitial pneumonia) were remarkable findings due to mycoplasmal infection which brings about immune suppression, ciliary dysfunction and unregulated production of TNFα. (MacGavin et al., 2001 and Shahr-
The appearance of multiple abscesses in the pulmonary tissue is a direct consequence of impairment of bacterial clearance by lysis of pulmonary macrophages and infiltrating leukocytes by *M. haemolytica* leukotoxin as hypothesized by Bottone (1998). On the other side, Adegboye et al. (1995) regarded mycoplasma as one of the causative agents of bovine bronchopneumonia with abscessation. Fibrinous pleuritis with adhesion to the thoracic cavity occurs as extension of pneumatic mannheimiosis or mycoplasmosis as mentioned by Abo-El Leil (1992) and MacGavin and Zachary (2007). Many investigations stated that BVDV may infect lung tissue as it contributes to the pathogenesis of respiratory diseases through immunosuppression and acts synergistically with other respiratory pathogens to facilitate bacterial colonization and gave rise to pneumonia. (Jubb et al., 1993; Hosny et al., 1996; Brodersen and Kelling, 1998; Aly et al., 2003 and Loneragan et al., 2005).

Reggiardo and Kaeberle (1981); Haines et al. (2004) and Gagea et al. (2006b) pointed out that BVDV infection interfered with normal clearance mechanisms due to leucopenia since, animals with deficient humeral and cell mediated immunity die due to impairment of polymorphnuclear cell functions.

The liver in most cases exhibited hemorrhages, hepatocellular degeneration and necrosis and vasculitis. These entities may be attributed to toxemia produced by *M. haemolytica* (Jones et al., 1997). Furthermore, mycoplasma infection brings about necrotic foci in the hepatic parenchyma due to thrombosis of the hepatic blood vessels.

Bacterial colonization was evident in the hepatic blood vessels and sinu-soids and in between degenerated hepatocytes. A picture which related to immunosuppressive effect of BVDV which lead to ascending of bacteria from disrupted gastrointestinal tracts as reported by (David et al., 1994; Piero et al., 1997 and Ibtisam et al., 2000).

The heart muscles of dead calves displayed focal hemorrhages and flappyness of cardiac-myocytes which appreciated microscopically as vaculations and necrosis of the cardiac myocytes. Similar findings were previously recorded by David et al. (1994), and they accentuates the hypothesis of Piero et al. (1997) and Haines et al. (2004) who detected BVD viral antigens in pukinji fibers, ventricular muscles and medium sized arteries and suggested that these lesions may promote arrythmogenesis.

The kidneys revealed variety of pathological lesions emphasized by proliferative and membranous glomerulonephritis, necrosis of the tubular epithelium and plasmacytic, lymphocytic interstitial infiltration.

The emphysis of proliferative and membranous glomerulo-nephritis which represented the anatomic forms of immune complex glomerulonephritis in-
cited by BVDV infection as hypothesized by Jubb et al. (1993) and MacGavin et al. (2001) who mentioned that BVDV infection brings about increase in the mesangial cells and incriminated this infection as one of the causative agents of immune complex glomerulonephritis.

Owing to the fact that BVDV induces a complex response by activating cells mediating the cellular and humoral immune response where antigen antibody complex formed in the circulation trapped in the glomeruli and induce glomerular damages (MacGavin et al., 2001 and Kumar et al., 2005).

Jubb et al. (1993) mentioned that the renal interstitial leukocytic infiltration is a sequence of glomerular lesions. Moreover, David et al. (1994); Ibtisam et al. (2000) and Aly et al. (2003) reported the same lesions in kidneys of dead calves infected with BVDV. In one dead calf, the renal cortex displayed periglomerular and interstitial fibrosis and atrophied hypocellular glomerular tufts. These histological features represented the signs of chronic glomerulonephritis as described by Jubb et al. (1993) and MacGavin et al. (2001). Dystrophic calcification was detected at some parts of the renal cortex. MacGavin et al. (2001) suggested that calcification of fibrotic kidney occurred due to alterations in calcium phosphorus metabolism associated with chronic renal failure.

The detection of numerous foci of degenerated or necrotic epithelial cells in the stratum spinosum in the ruminal and/or reticular mucosa was recognized in dead calves which may progress to erosion and/or ulceration, Since BVDV targets the stratified squamous epithelium of the upper alimentary tracts. (David et al., 1994, Bodersen and Kelling, 1998; Ibtisam et al., 2000; Aly et al., 2003 and Amiridis et al. 2004). The intestinal tracts of dead calves exhibited severe congestion on both serosal and mucosal surfaces with numerous erosive and/or ulcerative lesions. These pictures were reported by many authors (Ibtisam et al., 2000 and Aly et al., 2003).

Histologically, there were various alterations manifested by necrotizing enteritis, inflammatory cell infiltration and fibrinoid necrosis and vasculitis of some submucosal blood vessels. MacGavin and Zachary (2007) explored out that BVDV gain entrance to the intestinal mucosa through M cells (mem-branous or micro-folded surface cells) which serves as a portal of entry of some pathogens.

The necrotic lesions of the surface and cryptal enterocytes with exfoliation into the intestinal and cryptal lumina were explained by Mac-Gavin et al. (2001) who pointed out that necrotizing processes initiated at the gut associated lymphoid tissue with extension to the over lining epithelium.

These lesions were in attribute to potentiation of BVDV to the epithelial linings of the alimentary tracts as de-
scribed by Jubb et al. (1993); David et al. (1994); Brodersen and Kelling (1998); Ibtisam et al. (2000) and Aly et al. (2003).

Extensive damage of the cryptal epithelium leads to collapse of the lamina propria with subsequent ulceration which considered one of the diagnostic lesions of BVDV infection (Ibtisam et al., 2000 and Aly et al., 2003 and MacGavin and Zachary, 2007).

Hyperplasia of globlet cells was evident in some intestinal glands which represented a feature of inflammatory conditions of the intestine as mentioned by Jones et al. (1997).

One of the most important microscopic lesion in the submucosal blood vessels was fibrinoid necrosis and vasculitis which occurred due to increased vascular permeability that lead to insudation of fibrinogen into the subendothelial area and initiates inflammatory condition as suggested by Kumar et al. (2005). A finding which accentuates the hypophesis of Jubb et al. (1993), who mentioned that BVDV infected endothelial cells of intestinal blood vessels and brings about vasculitis.

One of the pathological hall marks in the spleen and mesenteric lymph nodes was a prominent lymphofollicular depletion as well as depletion of lymphoid cells in the cortical and paracortical zones. Since BVDV targets lymphocytes and brought about necrosis and destruction of lymphoid cells in the lymphoid follicles and many studies recorded perturbations of the immune system by BVDV (Bordersen and Kelling, 1998; Ibtisam et al., 2000 and Aly et al., 2003)

Moreover, the mesenteric lymph nodes showed widely dilated lymphatic sinuses with the presence of erythrocytes admixed with histiocytes and lymphocytes. Jubb et al. (1993) mentioned that erythrocytes delivered to lymphatic sinuses in animals suffered from toxemic conditions. Meanwhile sinus histiocytosis was evidenced in some mesenteric lymph nodes. This specific entity indicate antigenic stimulation of the node as mentioned by Jones et al. (1997) and consistently correlated with Kumar et al. (2005) who speculated that sinus histiocytosis occurred in lymph nodes with continuated destruction of its cells. Chronic lymphadenitis which detected in one dead calf represented the end stage of various inflammatory conditions of the node as suggested by (MacGavin et al., 2001).

CONCLUSION

Mortalities in imported calves may be attributed to mixed infection of viral, mycoplasmal and bacterial infection (M. haemolytica).

Fibrinous pleuropneumonia was observed in this study, which considered as a complex disease observed in imported animals leading to high mortalities.

Mycoplasma bovis and M. haemolytica with BVDV virus as immunosuppressive virus were considered as a
complicated infection in imported calves leading to fibrinous pleuropneumonia.

Therefore the purchased stocks must be screened and tested for virus, mycoplasma as well as bacteria and infected animals should be identified.

It so important to pay attention to the role of BVDV in imported calves as raising the possibility of existence of PI animals. Those animals may cause extensive shedding of the virus in the population and resulted in severe outbreaks with high mortalities. So, animals imported to Egypt should be free from BVDV infection, PI animals must be detected and removed by applying control program to all imported herds.

Antimicrobial susceptibility tests should be applied directly after laboratory diagnosis of *M. haemolytica* to avoid the misused of antimicrobial drugs and release of high multidrug resistant strains of *M. haemolytica*.

Environmental stress factors must be avoided by good ventilation and feeding, and over crowding during transportation.

REFERENCES


Aly, N. M.; Shehab, G. G. and Abd


infection, 10th Ed., Arnold oxford press, Ins. USA.


Bovine viral diarrhea.”


دراسات بكتيرية وميكوبلازمية وفيروسية وباثولوجية على حالات النفوق في الأبقار المستورة

فاطمة محمود محمد درويش*, سمية السيد أحمد الشافعي**, أميمة عبد العزيز شمس***، زينب رشدي محمد****
* قسم الباثولوجيا، ** قسم البكترىولوجيا، *** قسم الفيروولوجيا، **** قسم الميكوبلازما
- معهد بحوث صحة الحيوان بالدقى- جيزة

الملخص العربي

تم إجراء هذه الدراسة على حالات النفوق التي ظهرت في قطيعين من أبقار الفيروسات المستورة خلال شهرتين متتاليتين من الوصول إلى حجرة العين السخنة بمحافظة السويس في عامي 2006 و2007. وقد وجد أن نسبة النفوق في العينتين الأولى والثانية 80% ونسبة النفوق في القطيع الثاني 90%. أظهرت الحيوانات المريضة في كلا القطيعين أعراض تنفسية وآفاتات عينية وأنفية مع سيولة العين وامتلاء عن الظاهر واسهل مائي شديد.

تم إجراء الدراسة التشريحيه للمجلة النافية والبحث على عينات من الأعضاء الداخلية وكذلك من مسحة منعسة وعینية وشرجي م من العجلة المريضة وذلك لفحص البكتيرى والفيروسات وميكوبلازما والفحص الباثولوجي. وبياضة لفحص على عينات من المصل وعينات دم على أسطح للجلد من كل من العجلة المريضة والسلمية لفحص الفيروكو والسيرولوجي.

ومن خلال الفحص البكترى وجد أن اختيار الأجار الترمسيي واختيار الاليزا من أسرع وأدق الاحزاب التعرف على أجزاء الفيروسات السهل الاقترار البكترى. وقد تم عمل الفحص على عينات الدم والمسحات الأنفية من عينات الدم والمسحات الأنفية وترشح من عينات الدم والمسحات الأنفية بعد ترشح العينات من عينات الدم والمسحات الأنفية والشريحة من العجلة المريضة وأيضاً من العجلة الداخلي للعجلة بالنقاط. وقد تم التعرف عليه باستخدام الفحص النائي باستعمال مجهزات مرجعية الفيروسات. وأيضاً تم تجميع عينية مصل من عينات سلمية من عينات سلمية من الحيوانات المريضة واختبارها باستخدام اختبار التخلرس البكترى للتنبؤ على الأجسام المتحية للعجلة الفيروسية.

ومن خلال الفحص البكترى وجد استخدام اكسيموليكا هيموليتينا (الاستييلا هيموليتينا) من الورن والركا والكلك وكذلك من المسحات الأنفية. العينات المعزولة من كريتتيكا إلى النوع (أ) وال نوع (ب) وقد تم إجراء اختبار الضراوة عند 200% وجد أنها شديدة الضراوة حيث أحدثت نفوق في الجراثيم في خلال 24 ساعة. وباشراء اختبار الاستجابة للمعزولة، وجد أن معظم العينات معزولة شديدة المقاومة لمجموعة المعزولة الميكوبلازمية (المتابوليك، حامض الناقلديسيميك، أوكسيتراسيكيلين، والدوزاكس) وشديدة الحساسية لكل من نورفوكاسليسين، أميبسولين، والرستروميسين.
وبالفحص الميكوبلازمي فقد تم عزل ميكرونب الميكوبلازمـا بوفيز من القصبة الهوائية والرئة والكبد للحيوانات الناقفة وكذلك من (Mycoplasma bovis) المسحات الأنفية والعينية من الحيوانات المرضية.

ومن خلال الفحص الهستوپاثولوجي أظهرت العجول الناقفة التهاب رنوا شعبي فيبريني بيني وتتكزر خلايا الكبد مع استمالها وتتكز بعضا القلب مع وجود التهاب مناعي بالكلى تحورى وغشائي. أظهر كلا من الطحال والغدد الليمفاوية المساوقة نقصا في الخلايا الليمفاوية وظهرت أيضا استحالا وتتكز بخليج الإبرة النسيج الطلائي للكرش والشبكية كما ظهر التهاب معوي تتكرر مع بؤرا من التأكل والتقرح في النسيج الطلائي للإمام.

أوضحث الدراسة ان السبب الرئيسي للفوق هو العدوى بفيروس الاصهال البقرى الذي مهم وساعد تعاوننا المبتهج وعُلي وموكز واسكو بوفيز عل احداث التفوق وذلک من خلال تأثيره المثبط للمناعة.

المحكون:

أ.د. أحمد محمد عمار
أ.د. عفاف أحمد الغواص
أ.د. نوال محمد علي

استاذ الميكروبيولوجي - كلية الطب البيطري - جامعة الزقازيق
استاذ الباثولوجي - معهد بحوث صحة الحيوان - الدقى
استاذ الفيروجيا - معهد بحوث صحة الحيوان - الدقى