A Retrospective Discussion of Three Infectious Agents as Possible Causes of Sudden Deaths in a Flock of Small Ruminants

[1]Angeliki R Burriel, [1,2]Charalabos Billinis and [3]Vassiliki Spyrou
[1]Faculty of Veterinary Medicine, University of Thessaly,
Trikalon 224, Karditsa 43100, Greece
[1,2]Institute of Biomedical Research and Technology,
Papanastasiou 51, 41222, Larissa, Greece
[3]Department of Animals Production, Technological
Education Institution, 41110, Larissa, Greece

Abstract: In a flock of 332 sheep and goats, 120 (90 adults and 30 youngsters) died within 45 days. The clinical and post-mortem examinations were indicative of a contagious disease, thus prompted the isolation of the flock by the State Veterinary Services and the in-depth laboratory investigation of the possible causes of death. Tissue and blood sample examination confirmed suspicion of *Clostridium perfringens* involvement but also involvement of malignant catarrhal fever virus of cattle and respiratory cyncytial virus. Samples examined were negative for *Leptospira* sp. peste des petits ruminants virus and blue tongue virus.

Key words: *Clostridium perfringens,* MCF, RCV, sheep, deaths, suspicion

INTRODUCTION

Sheep and goat losses are prevented by management practices, appropriate vaccination schemes and prompt laboratory diagnosis for defining the specific measures recommended to control the further development of problems. The causes of deaths of greatest concern to farmers vary between countries because they depend on the prevalence of deadly infections, the mode of transmission and the farming systems practiced within a country. Some infections of economic concern to all small ruminant farmers worldwide are blue tongue virus, foot and mouth virus, pox virus, peste des petits ruminant virus, small ruminant lentiviruses, enterotoxaemia and others (Singh and Prasad, 2008; Singh *et al.*, 2004; Schwartz-Cornil *et al.*, 2008; Uzal and Songer, 2008; Rea-Boutrois *et al.*, 2009).

The threat of income loss from such hard to control causes, regardless of management practices or diagnostic means available, encourages farmers to immediately report deaths to their veterinary surgeon. However, timing and available means to make an immediate diagnosis, thus arrive at specific measures of preventing the worsening of an observed condition could determine the livelihood of a farmer or even a country. Many deaths at once or within a short time are hard to manage effectively and timely, especially if they are resulting from a viral infection spreading fast between farms and countries. Mixing of species (sheep and goats), introduction of new arrivals without isolation, the timing of changes in nutrition are some areas that farmers should routinely observe and manage for preventing the fast spreading of unusual infectious causes of deaths (Schwartz-Cornil *et al.*, 2008; Rea-Boutrois *et al.*, 2009). The present study discusses some probable causes of sudden deaths occurring within a period of 45 days.

MATERIALS AND METHODS

Flock investigated: The flock had at the time of experiencing the first death 255 ewes, 19 does, 56 lambs and two kids. Within a period of 45 days 90 adults of which 26 pregnant and 55 at lactation 28 lambs and two kids died. All adults had been vaccinated with the Dialuen P (Intervet Ltd, Netherlands) 1 and 6 months prior to the occurrence of the first death. The flock was intensively managed, thus housed permanently and fed concentrates and roughage supplied by the same commercial source for the past 5 years. The flock was freely moving around a yard of about three acres. Three of the 19 goats had been introduced in the flock about 60 days prior to the occurrence of the first death and they were burren. The source of goats was state certified.

Corresponding Author: A.R. Burriel, Faculty of Veterinary Medicine, University of Thessaly, Trikalon 224, Karditsa 43100, Greece

History of deaths: The first three deaths (two pregnant ewes and one pregnant goat) occurred within 5 days and were sudden (no prior clinical symptoms had been observed by the farmer). At the same time, two adults (one ewe and one goat) aborted. The farm's veterinarian was called at the occurrence of the third death, performed a necropsy and recorded post-mortem findings indicative of enterotoxaemia. No samples for laboratory examinations were collected at this time but appropriate antibiotic and supportive treatment for all animals was recommended. The enclosed facilities of the farm were disinfected but deaths continued. Animals died suddenly or showed shortly prior to death, anorexia, lethargy, tremor, hunched backs and sudden drop of milk production. The continuance of deaths and the appearance of clinical signs forced the farmer to seek the support of the State Veterinary Services. History of the flock and the *in situ* examination of dead and sick animals confirmed prior conclusions but they also rased suspicion of a possible viral infection such as bluetongue virus. The flock was thus, put on isolation while treatment continued, including the multivalent vaccine Covexin (Schering-Plough, USA) administered to all remaining animals. The government veterinarians collected for laboratory examination tissue from parenchymatic organs, serum samples from animals showing clinical signs and samples of water and feed stuffs. Despite treatments and management measures undertaken (separation of clinically ill animals, goats from sheep, pregnant from lactating), deaths continued reaching 120 in about 45 days.

Post-mortem findings: According to farm records filled by the state veterinary staff, the post-mortem observations showed sever haemorrhages of the digestive system, lungs and lymph nodes, fibrinous pericarditis and a large ecchymotic lesion at the base of pneumonic artery.

Examination of samples collected: Serum samples collected from 15 sick animals were tested with the standard operating procedures of the Institute of Infectious and Parasitic Diseases, State Veterinary Institutes, Ministry of Agriculture, Athens, Greece for antibodies to nine serotypes of *Leptospira* sp. blue tongue virus, peste des Petits Ruminants Virus (PPRV), Bovine Respiratory Cyncytial Virus (BRCV) and Malignant Catarrhal Fever virus of bovine (MCF).

Tissue samples were cultured for aerobic and anaerobic bacteria following standard operating procedures and examined by PCR for the detection of bluetongue virus. They were also examined, together with water and feed samples, by the Veterinary Toxicology Laboratory, State Veterinary Institutes, Ministry of Agriculture, Athens, Greece for various toxic agents using standard operating procedures.

Thirty samples of each, blood, saliva and nostril excretions were examined by PCR for the presence of BRCV and MCF. RNA extraction from the samples for BRCV was possible using Trizol reagent (Invitrogen) and following the manufacturer's instructions. Contamination by foreign ribonucleases was prevented by following the recommendations of Sambrook *et al.* (1989). Reverse-Transcription (RT) reactions were performed by mixing 7.6 μL of RNA extraction product with 12.4 μL of an RT premix to obtain a final concentration of 1 × first strand buffer (Invitrogen), 0.5 mM dNTP mix (Invitrogen), 10 mM DTT (Invitrogen), 100 U of Moloney Murine leukaemia Virus (MMTV) reverse transcriptase (Invitrogen) and 3.5 pmol L^{-1} of random hexamers (Amersham Bioscience) per reaction. Following preparation of the RT reaction mixtures on ice, reverse transcription reactions were carried out at 37°C for 30 min and were terminated by heating for 5 min at 95°C. PCR amplifications were carried out observing the guidelines described by Kwok and Higuchi (1989). Each PCR reaction mixture contained 2 μL of RT product, 1 ×PCR buffer (Invitrogen), 1 mM $MgCl_2$ (Invitrogen), 0.2 mM of a dNTP mix (Invitrogen), 0.15 U of Taq Polymerase (Invitrogen) and 30 pmol of the primers G2.5 and F2.7 (Valarchet *et al.*, 2000). The reaction volume was adjusted to 20 μL with DEPC treated H_2O. The PCR mixtures were prepared on ice and the reactions were initiated by heating for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1.5 min at 72°C. The mixtures were then brought to 72°C for 5 min held at 4°C. From the last preparation, 10 mm were taken to perform the second round of PCR using the same mixture except for the substitution of internal primers VG1 and VG2 (Valarchet *et al.*, 2000).

The PCR reactions were initiated by heating for 5 min at 94°C, followed by 35 cycles of 45 sec at 94°C, 1 min at 58°C and 1 min at 72°C. The mixtures were again brought to 72°C for 5 min and held at 4°C. Following the second amplification, 10 μL of each PCR product was analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide (0.5 μg mL^{-1}). A 100 bp DNA ladder (Invitrogen) was analyzed on the same gel to serve as a size marker. The expected size of the PCR product was 541 bp. The PCR products were gel-purified (QIAquick GEL Extraction Kit, Qiagen Ltd.) and sequence analysis was performed twice (MWG Biotech, Ebersberg, Germany)

using forward and reverse nested-PCR primers. A phylogenetic and molecular evolutionary analysis was performed using MRGA 3.1 program (Kumar *et al.*, 2004).

A semi-nested PCR was performed for OvHV-2, as previously described (Baxter *et al.*, 1997). DNA was isolated from samples using a commercial extraction DNA isolation blood kit (Puregene, Gentra Systems, Minneapolis, Minnesota, USA) following the manufacturer's instruction. The semi-nested PCR for the detection of OvHV-2 sequences was conducted by the two-step amplification reaction cycles (Baxter *et al.*, 1997). Following the second amplification, 10 µL of each PCR product was analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide (0.5 µg mL^{-1}). A 100 bp DNA ladder (Invitrogen) was analyzed on the same gel to serve as a size marker. The expected sizes of the PCR products were 422 and 238 bp for the first and the second amplification, respectively.

RESULTS AND DISCUSSION

Negative were the PCR and the serologic examination for bluetongue virus, the serologic examination for PPRV and *Leptospira* sp. and all toxicology examinations.

Positive were 15 serum samples for MCF and 12 of 15 for BRCV. The only microorganism isolated from parenchymatic organs was *Cl. perfringens*. The present discussion was prompted after the refusal of the Greek State Farmers' Insurance Agency refused compensation to the farmer. Compensation was refused on the ground that deaths due to *Cl. perfringens* are preventable and MCF and BRCV are not included by the Greek State in the list of death causes for small ruminants.

Thus, the farmer commissioned 4 months after the occurrence of deaths, additional scientific assistance from the Laboratory of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Thessaly. Laboratory staffs decided after reviewing available records to perform PCR for detection of BRCV and MCF. None of the examined samples were positive to MCF and only three of 30 animals were positive to BRCV. Regardless of results, the laboratory findings, history of the flock and the recorded post-mortem examinations dictate the discussion of the possible role of *Cl. perfringens*, MCF and BRCV in the number of deaths observed in this flock.

The vaccination scheme and handling of the vaccine were according to the farmer's answers, as recommended by the producer. Thus, the handling of the vaccine did not appear as the cause for non protective immunity. The possibility of a non-vaccinal strain of *Cl. perfringens* to

be involved in the deaths was not investigated by the state laboratory for undetermined reasons. However, the severity of the necropsy findings shows the possible involvement of either a non-vaccinal strain or factors predisposing to the failure of the vaccine (Blackwell and Bulter, 1992; Valarchet *et al.*, 2000).

Such factors are changes in the feeding regime or the consumption of concentrates high in carbohydrates and protein substrates, found to help the rapid multiplication of the microorganism (Uzal and Kelly, 1996) but they were not apparent in this farm. The feeding regime was recommended by the nutritionist of the feed stuffs supplier. Thus, the probable role of the two viruses detected by serology (and PCR) should be further discussed as possible contributors to the number of deaths and the severity of the condition observed.

Although, several months had passed and deaths and clinical signs had stopped, two questioned still needed to be discussed. One was if the two viruses could act as immunosuppressors and the other if they could have been the actual causes of death.

Interestingly, none of the three goats introduced to the flock died while they were according to the State Laboratories, serologically positive to MCF but not to BRCV.

MCF is considered a virus of cattle, the sheep being the asymptomatic maintenance host of the virus (O'Toole *et al.*, 1995; Taus *et al.*, 2007). However, some scientists believe that the virus is capable of causing clinical disease to all ruminants but causing severest disease in cattle, deer and buffalo (Imai *et al.*, 2001; Simon *et al.*, 2003). Furthermore, the virus is clinically affecting non-ruminant species, such as pigs (Syrjala *et al.*, 2006). The reported clinical signs of severely affected animals are fever and depression, nasal discharges, lymphadenitis, conjunctivitis, sever vasculitis, extensive epithelial necrosis, severe hemorrhagic diarrhoea and signs of an infected central nervous system (Simon *et al.*, 2003; Li *et al.*, 2005; Vikoren *et al.*, 2006), all present at the investigated flock. These clinical findings were in the past thought as the result of autoimmune damage to target tissue but they are recently believed as the direct result of the cell infection by the virus (Imai *et al.*, 2001; Simon *et al.*, 2003; Vikoren *et al.*, 2006). Thus, investigated deaths could have resulted from infection by MCF. Strains reported as more virulent are those coming from wild animals belonging to the type *Alcelaphine herpesvirus* 1 (AHV-1) (Baxter *et al.*, 1997; Taus *et al.*, 2007; Cunha *et al.*, 2008). Those coming from sheep (Hart *et al.*, 2007; Cunha *et al.*, 2008) and possibly goats (Li *et al.*, 2001) belong to *Ovine herpesvirus* 2 (OvHV-2). However, the latter has recently

been reported as a type capable of multiplying in sheep (Cunha *et al.*, 2008), thus easily spreading from animal to animal in a flock, confirming the results of earlier experimental infection giving a clinical condition similar to that in cattle (Buxton *et al.*, 1985) and the one observed in this flock. In the studies of Buxton *et al.* (1985), most sensitive were pregnant ewes, a condition existing among the animals of the present flock. Thus, there is an increased possibility that MCF was one of the actual causes of death. Its source could have been one or all of the newly introduced does. A new type of MCF has been recognized in goats (CpHV-2) (Li *et al.*, 2001) but is of unknown yet virulence. The use of PCR on samples collected from the flock >12 weeks from the mass deaths did not detect the virus, perhaps due to time lapse, intermittent shedding or inability of this PCR to detect the agent (Taus *et al.*, 2007). On the other hand positive serology for the BRCV was confirmed in the flock by PCR. The presence of this virus in the flock could have helped synergistically in the severity of the clinical manifestations of either or both, MCF and *Cl. perfringens*. The molecular typing of BRCV by PCR placed phylogenetically the strains into the group of French origin. What could, however be the role of this virus in the mass deaths of the flock? BRCV is a member of the genus Pneumovirus of the family Paramyxoviridae. It is thus, a virus of the respiratory tract, thought as an immunosuppressor (Woldehiwet and Sharma, 1992). It affects dendritic cells of the lungs, lymphocytes and macrophages (Meyerholz *et al.*, 2004; Fach *et al.*, 2007). Thus, cells also hosting the MCF virus. Generally, the infection of ruminants by BRCV is causing a mild respiratory disease associated mainly with the younger rather than the older animal (Meyerholz *et al.*, 2004).

However, infection may lead in severe disease if other immunosuppressive factors are also present or other pathogens are complicating the viral infection (Woldehiwet and Sharma, 1992; Brogden *et al.*, 1998). The laboratory evidence in the present flock support once more the role of mixed infections in the mass deaths observed in the flock. The two viruses helping, possibly, each other's excessive multiplication and both reducing the ability of the ewes' immune system to develop protective immunity against *Cl. perfringens* or effectively inhibit its excessive multiplication.

CONCLUSION

In the discussion of the case, the evidence support the very important role of the triple infection in the clinical findings, the proportion of deaths and the failure of the treatments applied to minimize losses.

REFERENCES

Baxter, S.I., A. Wiyono, I. Pow and H.W. Reid, 1997. Identification of ovine herpesvirus-2 infection in sheep. Arch. Virol., 142: 823-831.

Blackwell, T.E. and D.G. Bulter, 1992. Clinical signs, treatment and postmortem lesions in dairy goats with enterotoxemia: 13 cases (1979-1982). J. Am. Vet. Med. Assoc., 200: 214-217.

Brogden, K.A., H.D. Lehmkuhi and R.C. Cutlip, 1998. Pasteurella haemolytica complicated respiratory infections in sheep and goats. Vet. Res., 29: 233-254.

Buxton, D., H.W. Reid, J. Finlayson, I. Pow and E. Berrie, 1985. Transmission of a malignant catarrhal fever-like syndrome to sheep: Preliminary experiments. Res. Vet. Sci., 38: 22-29.

Cunha, C.W., D.L. Traul, N.S. Taus, J.L. Oaks, D. O'toole, C.M. Davitt and H. Li, 2008. Detection of ovine herpesvirus 2 major capsid gene transcripts as an indicator of virus replication in shedding sheep and clinically affected animals. Virus Res., 132: 69-75.

Fach, S.J., D.K. Meyerholz, J.M. Gallup, M.R. Ackermann, H.D. Lehmkuhl and R.E. Sacco, 2007. Neonatal ovine pulmonary dendritic cells support bovine respiratory syncytial virus replication with enhanced interleukin (IL)-4 And IL-10 gene transcripts. Viral Immunol., 20: 119-130.

Hart, J., M. Ackermann, G. Jayawardane, G. Russell, D.M. Haig, H. Reid and J.P. Stweart, 2007. Complete sequence and analysis of the ovine herpesvirus 2 genome. J. Gen. Virol., 88: 28-39.

Imai, K., T. Nishimoria, R. Horinob, K. Kawashimab and H. Muratab *et al.*, 2001. Experimental transmission of sheep-associated malignant catarrhal fever from sheep to Japanese deer and cattle. Vet. Microbiol., 79: 83-90.

Kumar, S., K. Tamura and M. Nei, 2004. MEGA 3.1: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinf., 5: 150-163.

Kwok, S. and R. Higuchi, 1989. Avoiding false positives with PCR. Nature, 339: 237-238.

Li, H., J. Keller, D.P Knowles and T.B. Crawford, 2001. Recognition of another member of the malignant catarrhal fever virus group: An endemic gammaherpesvirus in domestic goats. J. Gen. Virol., 82: 227-232.

Li, H., K. Gailbreath, E.J. Flach, N. Taus and J. Cooley *et al.*, 2005. A novel subgroup pfrhabinoviruses in ruminants. J. Gen. Virol., 86: 3021-3026.

Meyerholz, D.K., B. Grubor, S.J. Fach, R.E. Sacco, H.D. Lehmkuhl, J.M. Gallup and M.R. Ackermann, 2004. Reduced clearance of respiratory syncytial virus infection in a preterm lamb model. Microbes Infect., 6: 1312-1319.

O'Toole, D., H. Li, S. Roberst, J. Rovnak and J. Demartini *et al.*, 1995. Chronic generalized obliterative arteriopathy in cattle: A sequel to sheep-associated malignant catarrhal fever. J. Vet. Diagn. Invest., 7: 108-121.

Rea-Boutrois, A., S. Villet, T. Greenland, P. Mehlen, Y. Chebloune, G. Verdier and C. Legras-Lachuer, 2009. Small ruminant lentivirus Tat protein induces apoptosis in caprine cells *in vitro* by the intrinsic pathway. Virology, 383: 93-102.

Sambrook, J., E.F. Fritsh and T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York.

Schwartz-Cornil, I.P., P.C. Mertens, V. Contreras, B. Hemati and F. Pascale *et al.*, 2008. Bluetongue virus: Virology, pathogenesis and immunity. Vet. Res., 39: 39-46.

Simon, S., H. Li, D. O'Toole, T. Crawford and L. Oaks, 2003. The vascular lesions of a cow and bison with sheep-associated malignant catarrhal fever contain ovine herpesvirus 2-infected CD8+ T lymphocytes. J. Gen. Virol., 84: 2009-2013.

Singh, B. and S. Prasad, 2008. Modelling of economic losses due to some important diseases in goats in India. Agric. Econ. Res. Rev., 21: 297-302.

Singh, R.P., P. Saravanan, B.P. Sreenivasa, R.K. Singh and S.K. Bandyopadhyay, 2004. Prevalence and distribution of Peste des petits ruminants virus infection in small ruminants in India. Rev. Sci. Technol., 23: 807-819.

Syrjala, P., H. Saarinen, T. Laine, T. Kokkonen and P. Veijalainen, 2006. Malignant catarrhal fever in pigs and a genetic comparison of porcine and ruminant virus isolates in Finland. Vet. Rec., 159: 406-409.

Taus, N.S., D.R. Herndon, D.L. Traul, J.P. Stewart and M. Ackermann *et al.*, 2007. Comparison of ovine herpesvirus 2 genomes isolated from domestic sheep (*Ovis aries*) and a clinically affected cow (*Bos bovis*). J. Gen. Virol., 88: 40-45.

Uzal, F.A. and J.G. Songer, 2008. Diagnosis of clostridium perfringens intestinal infections in sheep and goats. J. Vet. Diagn. Invest., 20: 253-265.

Uzal, F.A. and W.R. Kelly, 1996. Enterotoxaemia in goats. Vet. Res. Comm., 20: 481-492.

Valarchet, J.F., F. Schelcher and H. Bouthy, 2000. Evolution of bovine respiratory syncytial virus. J. Virol., 74: 10714-10728.

Vikoren, T., H. Li, A. Lillehaug, M.C. Janassen, I. Bockerman and K. Handeland, 2006. Malignant catarrhal fever in free-ranging cervids associated with OVHV-2 and CPHV-2 DNA. J. Wildl. Dis., 42: 797-807.

Woldehiwet, Z. and R. Sharma, 1992. Evidence of immunosuppression by bovine respiratory syncytial virus. Scand. J. Immunol., 36: 75-80.