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ΤΜΗΜΑ ΚΤΗΝΙΑΤΡΙΚΗΣ

ΣΗΜΑΣΙΑ ΤΟΥ ΒΑΚΤΗΡΙΟΥ *SALMONELLA ENTERICA* SUBSP. *DIARIZONAE* ΣΤΟ ΔΙΑΡΡΟΪΚΟ ΣΥΝΔΡΟΜΟ ΤΩΝ ΑΡΝΙΩΝ

ΔΗΜΗΤΡΙΟΣ Χ. ΧΑΤΖΟΠΟΥΛΟΣ

Κτηνίατρος

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

που εκπονήθηκε στην Κλινική Μαιευτικής και Αναπαραγωγής
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Καρδίτσα 2019

Στη Στέλλα, την Κωνσταντίνα και τον Χρήστο,
για τον χρόνο που έπρεπε να βρίσκομαι μακριά τους.

ΠΕΡΙΛΗΨΗ

Η παρούσα διατριβή εστιάζεται στην αξιολόγηση του ρόλου του βακτηρίου *Salmonella enterica* subsp. *diarizonae* στην αιτιολογία του διαρροϊκού συνδρόμου των αρνιών και αποσκοπεί ειδικότερα: (α) στη μελέτη της έκτασης της μόλυνσης από *S. enterica* subsp. *diarizonae* σε εκτροφές προβάτων με κλινικά περιστατικά διαρροϊκού συνδρόμου των αρνιών και (β) στη μελέτη της παθογένειας της μόλυνσης από *S. enterica* subsp. *diarizonae* για τα αρνιά.

Η διατριβή χωρίζεται σε τρία κεφάλαια και ακολουθεί η Γενική Συζήτηση.

Στο Κεφάλαιο Ι, ανασκοπείται η σχετική βιβλιογραφία. Το Κεφάλαιο υποδιαιρείται σε τρία τμήματα. Στο τμήμα Α, ανασκοπείται συνοπτικά η βιβλιογραφία σχετικά με την ταξινόμηση και της ονοματολογία του γένους *Salmonella*. Στο τμήμα Β, ανασκοπείται η βιβλιογραφία η σχετική με τις λοιμώξεις από *Salmonella* στα πρόβατα. Στο τμήμα Γ, ανασκοπείται η βιβλιογραφία η σχετική με τις λοιμώξεις από *Salmonella enterica* subsp. *diarizonae* σε ζώα.

Στο Κεφάλαιο ΙΙ, μετά από σύντομη παρουσίαση του βακτηρίου *S. enterica* subsp. *diarizonae* στο πλαίσιο του διαρροϊκού συνδρόμου των αρνιών σε εκτροφές προβάτων (Τμήμα Α), παρουσιάζονται αρχικά ένα περιστατικό φυσικής μόλυνσης σε αρνιά που οφειλόταν στο εν λόγω βακτήριο (Τμήμα Β) και στη συνέχεια μία μελέτη στο πεδίο σχετικά με την έκταση της φυσικής μόλυνσης σε εκτροφές προβάτων στην Κεντρική Ελλάδα (Τμήμα Γ).

Σε μία εκτροφή προβάτων παρατηρήθηκαν αυξημένα κρούσματα διαρροϊκού συνδρόμου σε νεαρά αρνιά. Τα κρούσματα διάρροιας αφορούσαν σε αρνιά ηλικίας 5 έως 15 ημερών σε περίοδο 7 ημερών. Συνολικά εκδήλωσαν διάρροια 25 νεαρά αρνιά, ποσοστό που αντιστοιχούσε σε 15,6% των αρνιών που είχαν γεννηθεί στην εκτροφή (n = 160). Από αυτά, 10 είχαν ήδη πεθάνει πριν από την επίσκεψη στην εκτροφή (θνησιμότητα: 0,400). Κατά την επίσκεψη, πραγματοποιήθηκε κλινική εξέταση σε ασθενή αρνιά, τα οποία παρουσίαζαν έντονη διάρροια και νεκροτομική εξέταση σε πτώματα δύο αρνιών, με κυριότερα παθολογικά ευρήματα την απουσία περιεχομένου στον γαστρεντερικό σωλήνα, την παρουσία φλεγμονωδών αλλοιώσεων στον πυλωρό, τον ειλεό και το λεπτό έντερο και την παρουσία πολυάριθμων πετεχειών στο τοίχωμα του εντέρου, το οποίο φαινόταν πιο λεπτό από το φυσιολογικό. Ελήφθησαν δείγματα κοπράνων από τα ασθενή ζώα, για βακτηριολογική, ιολογική και παρασιτολογική εξέταση, τα αποτελέσματα των οποίων έδειξαν μόνον παρουσία *S. enterica* σε καθαρή καλλιέργεια. Το βακτήριο απομονώθηκε επίσης από ιστοτεμάχια λεπτού εντέρου και ήπατος από τα νεκροτομηθέντα αρνιά μετά από βακτηριολογική εξέταση. Τα στελέχη ταυτοποιήθηκαν πλήρως ως *Salmonella enterica* subsp. *diarizonae* με τον αντιγονικό συνδυασμό 61:k:1,5,(7).

Συνταγογραφήθηκε η θεραπευτική χορήγηση ενροφλοξακίνης στην εκτροφή. Μετά το τέλος της χορήγησης της αγωγής, πραγματοποιήθηκε συλλογή δειγμάτων κοπράνων από τα ζώα της εκτροφής επί 42 ημέρες, από τα οποία απομονώθηκε το βακτήριο 22 ημέρες μετά το τέλος της αγωγής.

Ακολούθως, πραγματοποιήθηκε διερεύνηση της παρουσίας του βακτηρίου σε δείγματα κοπράνων αρνιών (n = 119) με διάρροια σε 21 εκτροφές προβάτων στην Κεντρική Ελλάδα. Όλα τα δείγματα εξετάζονταν για την παρουσία *Salmonella* με βακτηριολογικές τεχνικές. Το βακτήριο δεν απομονώθηκε από κανένα δείγμα από καμία από τις 21 εκτροφές

Στο κεφάλαιο ΙΙΙ της παρούσας διατριβής παρουσιάζονται τα αποτελέσματα πειραματικής μόλυνσης για μελέτη της σημασίας του βακτηρίου *S. enterica* subsp. *diarizonae* στο διαρροϊκό σύνδρομο των αρνιών.

Συνολικά, 12 αρνιά ενοφθαλμίστηκαν με *S. enterica* subsp. *diarizonae*, ένα στέλεχος το οποίο είχε απομονωθεί από κλινικό περισταιτκό της μόλυνσης. Ο ενοφθαλμισμός πραγματοποιήθηκε με χορήγηση από το στόμα εναιωρήματος με $0,75 \times 10^9$ έως $1,8 \times 10^9$ c.f.u. του βακτηρίου. Ως αρνητικοί μάρτυρες χρησιμοποιήθηκαν 4 αρνιά. Σε τακτικά χρονικά διαστήματα, συλλέχθηκαν διαδοχικά δείγματα κοπράνων, αίματος και επιχρίσματος στοματικού βλεννογόνου από όλα τα αρνιά στη μελέτη. Στα ίδια χρονικά σημεία συλλέχθηκαν δείγματα κοπράνων και γάλακτος από όλες τις προβατίνες, μητέρες των ανωτέρω αρνιών. Τα αρνιά θανατώθηκαν διαδοχικά 1, 2, 4, 7, 10, 14 και 21 ημέρες μετά την έναρξη της μελέτης και συλλέχθηκαν δείγματα ιστών από το λεπτό έντερο, το ήνυστρο, το ήπαρ και τη χοληδόχο κύστη, για εξέταση με βακτηριολογικές τεχνικές για απομόνωση και ταυτοποίηση *Salmonella* και με μοριακές τεχνικές (αλυσιδωτή αντίδραση πολυμεράσης - PCR) για ανίχνευση του γονιδίου *invA* του βακτηρίου, καθώς και για ιστοπαθολογική αξιολόγηση. Συνολικά, απομονώθηκε *Salmonellla* από όλα τα ενοφθαλμισμένα αρνιά (1,000) και συνολικά από 45/77 σχετικά δείγματα κοπράνων (0,584), με διάμεση διάρκεια απομόνωσης 2,4 ημέρες μετά τον ενοφθαλμισμό. Το βακτήριο απομονώθηκε επίσης από επιχρίσματα στοματικού βλεννογόνου από 7 αρνιά (0,583) και συνολικά από 10/77 (0,130) σχετικά δείγματα, με διάμεση διάρκεια απομόνωσης 0,8 ημέρες μετά τον ενοφθαλμισμό. *Salmonella* απομονώθηκε επίσης από 2 διαδοχικά δείγματα γάλακτος από μία προβατίνα. Το βακτήριο απομονώθηκε από ιστοτεμάχια συνολικά 9 αρνιών: από το λεπτό έντερο 6 (0,500), το ήνυστρο 4 (0,333), το ήπαρ 3 (0,250) και τη χοληδόχο κύστη 2 (0,167) αρνιών. Το γονίδιο *invA* ανιχνεύτηκε σε δείγματα από όλα τα ενοφθαλμισμένα αρνιά, με διάμεση διάρκεια ανίχνευσης στα δείγματα κοπράνων 5,5 ημέρες και στα επιχρίσματα στοματικού βλεννογόνου 1,3 ημέρες. Το γονίδιο ανιχνεύτηκε επίσης σε 3 δείγματα γάλακτος από τις μητέρες των ενοφθαλμισμένων αρνιών. Κατά την ιστοπαθολογική εξέταση, παρατηρήθηκε ηνυστρίτιδα με υποεπιθηλιακή συσσώρευση λευκοκυττάρων. Στο λεπτό έντερο, οι αλλοιώσεις αφορούσαν σε

διάταση και οίδημα των επιθηλιακών κυττάρων, λευκοκυτταρική διήθηση και υπερπλασία των λεμφοειδών οζιδίων με εμφανή βλαστικά κέντρα αρχικά και ατροφία ή / και εκφύλιση του λεμφοειδούς ιστού του εντέρου με παρουσία λεμφοκυττάρων, πλασμοκυττάρων και εωσινόφιλων κυττάρων υποεπιθηλιακά σε επόμενο στάδιο.

Τα συμπεράσματα που προκύπτουν από τα ευρήματα αυτής της διατριβής, είναι τα παρακάτω.

(α) Το βακτήριο *S. enterica* subsp. *diarizonae* είναι ευκαιριακό παθογόνο του πεπτικού συστήματος σε αρνιά. Το βακτήριο μπορεί να προκαλέσει ήπιας βαρύτητας νόσο, με αποβολή του στα κόπρανα και σαφείς ιστολογικές αλλοιώσεις στα όργανα του γαστρεντερικού σωλήνα. Επίσης, μπορεί να απομονωθεί από εσωτερικά όργανα των προσβεβλημένων αρνιών. Το βακτήριο μπορεί να προκαλέσει διαρροϊκό σύνδρομο σε νεαρά αρνιά. Το βακτήριο μπορεί να μεταδοθεί μεταξύ ζώων, π.χ., μεταδίδεται από τα αρνιά στις προβατίνες κατά το θηλασμό. Η αποβολή του στα κόπρανα είναι διαλείπουσα. Το βακτήριο μπορεί να απομονωθεί από το γάλα προβατίνων με μολυσμένα αρνιά.

(β) Παρατηρούνται διαφορές στη βαρύτητα της λοίμωξης από *S. enterica* subsp. *diarizonae* μεταξύ ζώων και εκτροφών. Η ανοσοεπάρκεια των μολυσμένων ζώων μπορεί να είναι παράγοντας που επηρεάζει τη βαρύτητα της νόσου.

Με βάση την κείμενη νομοθεσία και μετά από σχετική απόφαση στη με αριθμό 54/06.07.2017 συνεδρίαση της Γενικής Συνέλευσης Ειδικής Σύνθεσης του Τμήματος Κτηνιατρικής του Πανεπιστημίου Θεσσαλίας, η συγγραφή της διατριβής έγινε στην αγγλική γλώσσα.

Δημοσιεύσεις σχετιζόμενες με την παρούσα διατριβή

Στις παρακάτω επιστημονικές δημοσιεύσεις παρουσιάζονται τμήματα της παρούσας διατριβής:

I. D.C. Chatzopoulos, L.V. Athanasiou, V. Spyrou, G.C. Fthenakis, C. Billinis (2013). Rotavirus infections in domestic animals. *Journal of hellenic veterinary medical Society* 64:145–160.

II. D.C. Chatzopoulos, S. Sarrou, N.G.C. Vasileiou, K.S. Ioannidi, E. Peteinaki, G. Valiakos, K. Tsokana, E. Papadopoulos, V. Spyrou, A. Giannakopoulos, A. Sbiraki, D. Lacasta, J.P. Bueso, L.V. Athanasiou, C. Billinis ,G.C. Fthenakis (2016) "Dissemination of intestinal pathogens between lambs and puppies in sheep farms" *Small Ruminant Research* 141:5-10.

ΤΡΙΜΕΛΗΣ ΣΥΜΒΟΥΛΕΥΤΙΚΗ ΕΠΙΤΡΟΠΗ

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SIGNIFICANCE OF *SALMONELLA ENTERICA* SUBSP. *DIARIZONAE* IN THE DIARRHOEIC SYNDROME OF LAMBS

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A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Work carried out at the Department of Obstetrics and Reproduction
of the Faculty of Veterinary Medicine of the University of Thessaly

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ABSTRACT

The present thesis focuses in the study of infection of lambs by *Salmonella enterica* subsp. *diarizonae*, by performing work in the field and in an experimental setting. The general objective of the thesis is to increase available knowledge regarding gastrointestinal infections of lambs, particularly caused by *S. enterica* subsp. *diarizonae*. Specific objectives of the thesis are as follows: (i) the investigation of the extent of infection of lambs by *S. enterica* subsp. *diarizonae* in sheep farms with clinically evident diarrhoeic syndrome in lambs and (ii) the evaluation of the potential pathogenicity of *S. enterica* subsp. *diarizonae* for lambs.

The thesis is divided into three chapters followed by the General Discussion.

In Chapter I, the relevant literature is reviewed. The Chapter is subdivided into three Parts. In Part A, the literature about the taxonomy and nomenclature of *Salmonella* is briefly reviewed. In Part B, the literature about *Salmonella* infections of sheep is presented. In Part C, the literature about the clinical conditions caused by *S. enterica* subsp. *diarizonae* in animals is appraised.

In Chapter II, after a brief note regarding *S. enterica* subsp. *diarizonae* in field studies in sheep farms (Part A), an outbreak of diarrhoeic syndrome in lambs caused by the organism (Part B) and a field investigation of its occurrence in sheep farms in Central Greece (Part C) are presented. An outbreak of diarrhoeic syndrome in lambs was investigated in a sheep farm. Specifically, frequent cases of diarrhoea in 5 to 15 day old lambs were reported by the farmer within a period of 7 days. In total, 25 lambs had been ill with diarrhoea (incidence rate: 0.156). Among these, 10 had already died when the investigation started (case fatality: 0.400). Clinical examination revealed severe diarrhoea in lambs. A detailed post-mortem examination was performed in two lambs, with salient findings being: evidence of inflammation in jejunum and ileum in the small intestine and presence of petechiae in the intestinal wall, which was thinner than normal. From live animals, faecal samples were collected for laboratory investigations by bacteriological, virological and parasitic techniques. The tests revealed only abundant growth of *S. enterica* in pure culture from small intestine and liver tissue samples. Complete identification of the isolates indicated these as *Salmonella enterica* subsp. *diarizonae* 61:k:1,5,(7) isolates. Enrofloxacin was prescribed for administration to lambs. Further faecal samples were collected for up to 42 days post-treatment. The organism was reisolated 22 days after end of the treatment course.

An investigation was performed in 21 sheep farms in central Greece through faecal sample collection from diarrhoeic lambs (n = 119). Samples were processed for potential recovery of *Salmonella*, using also enrichment techniques. In no case, any *Salmonella* spp. organism was isolated.

In Chapter III, an experimental study regarding the potential role of *S. enterica* subsp. *diarizonae* for the diarrhoeic syndrome of lambs is presented (Parts B and C).

In total, 12 lambs were challenged with the *S. enterica* subsp. *diarizonae* isolate recovered during the field study (Chapter II). The lambs were challenged orally on the first day after birth, with a dose ranging from 0.75×10^9 to 1.80×10^9 c.f.u. Sequential blood samples, faecal samples and buccal swabs were collected at regular intervals after challenge from lambs. Sequential faecal and milk samples were collected from the dams of the lambs. Lambs were sequentially euthanised 1, 2, 4, 7, 10, 14 and 21 days after challenge. Samples were processed by bacteriological techniques for isolation of the challenge organism; they were also subjected to examination by molecular techniques (PCR) for detection of the *invA* gene. Tissue samples (small intestine, abomasum, liver, gallbladder) harvested from all euthanised lambs were also subjected to bacteriological and molecular examinations and were processed for histopathological examination. *Salmonella* was isolated from faecal samples of all lambs (1.000) at least once and in total from 45/77 (0.584) post-inoculation samples; median duration of bacterial isolation was 2.4 days post-inoculation. *Salmonella* was isolated from buccal samples from 7 lambs (0.583) at least once and in total from 10/77 (0.130) post-inoculation samples; median duration of bacterial isolation was 0.8 day post-inoculation. *Salmonella* was not isolated from any faecal sample from any dam of inoculated lambs; it was isolated from two milk samples, collected from the same ewe, dam of an inoculated lamb, on two consecutive sampling occasions. Finally, *Salmonella* was isolated from tissue samples of 9 lambs: small intestine of 6 (0.500), abomasum of 4 (0.333), liver of 3 (0.250) and gallbladder of 2 (0.167) lambs. The *invA* gene was detected in samples from all inoculated lambs and from 3 of their dams. In all samples, of all tissues, that had yielded *Salmonella* at the microbiological examination, the *invA* gene was also detected (1.000). Median duration of post-challenge detection of the gene was 5.5 days in faecal samples and 1.3 days in buccal samples. Histopathological examination revealed abomasitis with subepithelial presence of eosinophils, lymphocytes and plasma cells, consistently observed in all lambs. In the small intestine, salient lesions included initially distension and oedema of intestinal villi, leucocytic infiltration and hyperplasia of lymphoid nodules with apparent germinal centres, which were followed at later stages by atrophy and / or degeneration of the lymphoid tissue of the intestine with marked subepithelial infiltration of lymphocytes, plasma cells and eosinophils.

General and more specific conclusions derived from the results of the present thesis are as follows.

(a) *S. enterica* subsp. *diarizonae* is an opportunistic gastrointestinal pathogen in lambs. The bacterium can cause a mild infection, with faecal shedding and definite histopathological changes in the gastrointestinal tract; the bacteria can be isolated from internal organs of infected animals. The bacterium can be a causal agent of the diarrhoeic syndrome of lambs. Faecal shedding of the bacterium is intermittent. Horizontal transmission of the bacterium between animals can be possible; it can be transmitted from lambs to ewes during sucking. Isolation of the organism from the milk of the dams of infected lambs is possible.
(b) Differences in severity of infection can be observed among animals and farms. Immunological condition of animals might be a determinant of the severity of disease.

Publications associated with the present thesis

The following scientific papers presenting facets of the present thesis, are available:

I. D.C. Chatzopoulos, L.V. Athanasiou, V. Spyrou, G.C. Fthenakis, C. Billinis (2013). Rotavirus infections in domestic animals. *Journal of hellenic veterinary medical Society* 64:145–160.

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Infections of lambs

General considerations

Neonatal infections constitute a significant threat to newborns. At the early stage of life, newborns have to successfully respond to the transition from the 'protected' uterus to the external environment. Various pathogens can invade, proliferate and cause disease, with localised or systemic effects, often also death. Infections of newborn lambs have significant impact in sheep farms, regardless of production type and management system.

In sheep, the term 'neonatal infections' has been employed to describe problems caused by microbial or parasitic agents up to the 28th day of life (Bernal 2001, Dwyer 2008, Fragkou et al. 2010). In that species, the placenta allows minimal transfer of immunoglobulins from pregnant ewes to their embryos. Hence, immune cover of newborn lambs depends particularly upon uptake of colostrum to provide passive defensive ability to newborns.

The intestine of newborn lambs allows absorption of immunoglobulins for up to 48 hours after birth. In general, absorption of immunoglobulins through colostrum is progressively reduced with time (within this 48 hour frame), although, if no adequate quantity of immunoglobulins had been absorbed, then absorption can continue fully. Failure to receive adequate amount of immunoglobulins by lambs (which provides passive immunity) greatly increases risk of infection of newborns.

Frequently, reasons for such failure include delayed uptake of colostrum or uptake of insufficient quantity or bad quality of colostrum, as the result of various causes, e.g., (a) mammary diseases or other peri-parturient diseases (e.g., pregnancy toxaemia) of dams, (b) weakness of lamb or ethological reasons leading in delayed access to udder, (c) environmental (e.g., adverse weather) or management (e.g., overcrowded lambing areas) factors. A realistic target for neonatal lamb mortality rate in well-managed flocks should be 3% to 4%; the upper acceptable limit under any circumstances should be 5% to 7% (Fragkou et al. 2010; Mavrogianni et al. 2011). However, this target may not be met, with flocks having greater rates, in between 10 to 25% and occasionally even to 50% (Mellor και Stafford 2004, Sharif et al. 2005, Sargison 2008, Winter και Clarkson 2012, Scott 2015).

Apart from the obvious costs consequently to death of affected newborns, lamb infections also have further financial impact. These are as follows.

- Suboptimal growth of lambs.

- Consumption of increased amount of feed (and subsequently, inappropriate feed conversion ratio).
- Increased need for care of affected animals (labour, infrastructure etc.).
- Veterinary expenses.

In general, most cases of neonatal deaths after infections of lambs occur during the first week of their life. Thereafter, a combination of factors (e.g., improved immunity) contributes to reducing mortality rate among affected animals.

The most frequent infections of lambs refer to diseases of the gastrointestinal tract or the respiratory system. Other infections occurring less frequently include tetanus, hepatic necrobacilossis ('liver abscess syndrome'), arthritis of streptococcal aetiology, contagious ecthyma ('orf') and bacterial encephalitis.

Infections of the gastrointestinal tract

Infections of the gastrointestinal tract of lambs, caused by microbial or parasitic agents, are diagnosed in every sheep farm. Often, the various pathogens causing infections may co-exist in affected hosts, that way leading to problems in accurate aetiological diagnosis of the problem. Moreover, general predisposing factors are common among the various specific diseases (Giadinis et al. 2011). These include the following (Giadinis et al. 2011).

- Timely uptake of adequate amount of good quality colostrum.
- Overcrowding of animals in farm buildings during the lambing season.
- Suboptimal hygiene conditions in the farm
- Selenium deficiency, which contributes to inefficient immune response by lambs.

In view of the above, often, the various microbial or parasitic infections of lambs are collectively referred to as 'diarrhoeic syndrome of lambs', a term used independently of the causal agent of the problem and referring to a wide range of clinical signs. Moreover, approaches for control (therapeutic and prophylactic) of the syndrome includes generic actions and steps, which should be applied independently of the causal agents (Giadinis et al. 2011).

Escherichia coli infections are the predominant cause of gastrointestinal disorders in young lambs. Most *E. coli* isolates are non-pathogenic commensal residents of the intestinal lumen, potentially protecting the host from other more harmful organisms. Various factors may trigger their uncontrolled proliferation into the intestinal tract, leading to disease. Occasionally, *E. coli* isolates may also show invasive properties and translocate to tissues and organs outside the gastrointestinal tract. *E. coli* has been associated with two forms of disease: intestinal and septicemic (Radostits et al. 2007). Watery mouth disease is another clinical condition mostly associated with *E. coli* infection

mainly in newborn lambs, although various other factors (e.g., management) are also implicated in its aetiology.

Bacteria of the genus *Clostridium* (*C. botulinum*, *C. haemolyticum*, *C. novyi*, *C. perfringens*, *C. septicum*, *C. sordelii*, *C. tetani*) (Lewis 2007) are ubiquitous in sheep farms. They are also present in the gastrointestinal tract of healthy sheep. The organisms normally produce small quantities of toxins, which are tolerated by the host, without causing any sign of disease (Lewis 2007). Under certain conditions (e.g., inappropriate feeding regime), they can release large amounts of toxins, which are usually associated with various, often severe, clinical signs and increased mortality (Lewis 2007, 2011). Most clostridial-associated disorders in lambs are attributed to *C. perfringens*, which produces various toxins (*α*-, *β*-, *ε*-, *θ*-, *ι*-toxin) (Lewis 2007). The main gastroinstestinal problems associated with clostridia in lambs include enterotoxaemia, lamb dysentery, haemorrhagic enteritis and abomasitis-toxaemia (Lewis 2007).

Among parasites, cryptosporidia (mainly *Cryptosporidium parvum*), *Giardia* spp. and various coccidian species (mainly *Eimeria crandallis* and *Eimeria ovinoidalis*) also participate as aetiological agents of the diarrhoeic syndrome. Infected lambs may be presented with a variety of symptoms, these include diarrhoea or passing soft faeces with presence of mucus. After 3 to 4 days of diarrhoea, lambs become depressed and reluctant to suck, while a significant proportion of affected animals may die, consequently to severe dehydration. The disease may last for up to 10 days. In flocks with increased contamination, relapses may take place frequently.

The role of viral infections in the diarrhoeic syndrome remains unclear. Many viruses have been associated with intestinal disease in lambs. These include mainly rotaviruses, adenoviruses and coronaviruses, which are the ones most frequently detected; other viruses (e.g., astroviruses, bunyaviruses, caliciviruses, picornaviruses) can also cause occasional problems (Martella et al. 2015). Young animals, more often those younger than 14 days, are more likely to be affected (Martella et al. 2015). The salient clinical sign of the disease is diarrhoea; diarrhoeic faeces are white, yellow or, in severe cases, blood-tinted or haemorrhagic. Other clinical signs include anorexia, vomiting, depression and acute abdominal pain. Overall morbidity rates in an affected flock may be very high, reaching up to 60% of lambs therein, with mortality rates ranging from 10% to 30%.

Objective of the thesis

Gastrointestinal infections of lambs are a common clinical problem in newborn and young lambs. They may be caused by various organisms, some of these little-studied thusfar. Distribution and prevalence of the various pathogens vary depending on management, animal or environmental

factors. Further, in many cases, co-infecting pathogens may act synergistically, leading to aggrevated effects. All the above contribute to increased complexity of the pathogenesis of the syndrome.

The present thesis focuses in the study of infection of lambs by *Salmonella enterica* subsp. *diarizonae*, by performing work in the field and in an experimental setting. The general objective of the thesis is to increase available knowledge regarding gastrointestinal infections of lambs, particularly caused by *S. enterica* subsp. *diarizonae*. Specific objectives of the thesis are as follows.

- The extent of infection of lambs by *S. enterica* subsp. *diarizonae* in sheep farms with clinically evident diarrhoeic syndrome in lambs.
- The potential pathogenicity of the *S. enterica* subsp. *diarizonae* for lambs.

The present thesis has been carried out at the Department of Obstetrics and Reproduction of the Veterinary Faculty of the University of Thessaly. Further, laboratory work described in the thesis has been performed also at the Department of Microbiology and Parasitology of the Faculty. Research work started in 2014, with various stages performed until the beginning of 2019; it was followed by analysis of results and writing up of the thesis. The thesis was financially supported by departmental funds, as well as by funds of the Department of Microbiology and Parasitology of the Faculty.

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CHAPTER I

REVIEW OF THE LITERATURE

A. TAXONOMY AND NOMENCLATURE OF *SALMONELLA* SPP.

Introduction

Bacteria of the genus *Salmonella* were first isolated from pigs. They were described, in 1885, by D.E. Salmon and T. Smith as 'hog cholera bacillus' or 'bacillus cholera suis' (Salmon and Smith 1885). In 1900, the organism was renamed to *Salmonella choleraesuis*, in honour to D.E Salmon. The genus *Salmonella* is classified in the Enterobacteriaceae family and includes straight, rod-shaped, Gram-negative bacteria.

The bacteria can grow at an extended temperature range (6 - 45 ^{o}C), the optimal growth temperature being at 35 to 37 ^{o}C. Colonies of these bacteria in non-selective media are usually rough, 2 to 4 mm in diametre and of white to grey colour. Most species in the genus are motile. The organisms are non-spore forming, aerobic or facultative anaerobic. The various biochemical reactions of salmonellae can vary widely among the various species and subspecies.

Salmonella isolates abound in nature and can remain stable in environmental conditions, maintaining their virulence ability for months to years. In general, the bacteria are susceptible to ordinary disinfectants (pH < 3.8, water activity < 0.94) and sensitive to moderate heating (temperature > 70 ^{o}C).

Taxonomy

Despite recognition of *Salmonella* spp. as a pathogen of significant impact in human and animal health, classification of the organism still has not been fully agreed and remains under debate. Since its first isolation, several different taxonomic systems have been suggested by researchers. Most of these schemes were incomplete, leading to false, inaccurate or indistinguishable separation among salmonellae.

In the early stages of τηε study of *Salmonella* spp., each isolate was classified and named based on three main criteria: (1) clinical features of respective disease (*S. typhi* in humans, *S. typhimurium* in mice, *S. choleraesuis* in pigs), (2) host species predominantly affected by an isolate (*S. pullorum*) and (3) location where the initial isolation took place (*S. Newport*, *S. Panama*, etc).

However, it was quickly understood that many isolates could cause identical or similar clinical conditions in many species. Thus, the above approach has been quickly abandoned.

In the following years, two different systems for *Salmonella* spp. classification were developed and subsequently they evolved in parallel. The first was originally introduced by Kauffman (1966), based on antigenic composition of each isolate. According to that, the three major antigens of the organism (O antigens, H antigens, Vi antigens) were able to induce differing immune responses by the host and therefore serological detection could be achieved. In practice, serological classification was performed using specific antisera and isolates were thus differentiated by their serotyping results. Based on this scheme, salmonellae were distinguished in 'serotypes', or else called 'serovars', and each single serotype was considered as a unique *Salmonella* species.

The second taxonomy system was suggested by Borman et al. (1944) and it was based on separation of the *Salmonella* genus into three different species (*S. choleraesuis, S. typhosa* and *S. kauffmannii*). The main criterion for this separation was the different biochemical properties of each *Salmonella* isolate. Although this initial recommendation was generally ignored, later, several similar schemes were presented (Kauffmann and Edwards 1952, Ewing 1972). Among these, Le Minor et al. (1970) proposed that the four *Salmonella* subgenera (I, II, III and IV [Kauffmann and Edwards 1952]) should be considered as separate species. In this context, the term *Salmonella kauffmannii* has been adopted for salmonellae previously classified into subgenus I, *S. salamae* for those classified into subgenus II, *S. arizonae* for those classified into subgenus III and *S. houtenae* for those classified into subgenus IV.

In 1973, a decisive step in the taxonomy of the pathogen was performed by Crosa et al. (1973). Based on DNA-DNA hybridisation, it has been demonstrated that all *Salmonella* species were highly related to each other and therefore should have been classified into a single species. This unique *Salmonella* species was then assigned the name *Salmonella choleraesuis* (Skerman et al. 1980). However, the term 'choleraesuis' has caused several misunderstandings, as it was being used to describe both a *Salmonella* species and a specific salmonella serotype. In 1986, the International Committee on Systematic Bacteriology recommended that the type species for *Salmonella* would be changed to *Salmonella enterica* (Ewing 1986, Penner 1988). Le Minor and Popoff (1987) proposed the renaming of *S. choleraesuis* to *S. enterica* and its further classification to seven subspecies, as follows: (i) subspecies I: *Salmonella enterica* subsp. *enterica*, (ii) subspecies II: *S. enterica* subsp. *salamae*, (iii) subspecies IIIa: *S. enterica* subsp. *arizonae*, (iv) subspecies IIIb: *S. enterica* subsp. *diarizonae*, (v) subspecies IV: *S. enterica* subsp. *houtenae*, (vi) subspecies V: *S. enterica* subsp. *bongori* and (vii) subspecies VI: *S. enterica* subsp. *indica*. The division of subgenus III into IIIa and IIIb, was performed based on the genomic relatedness among isolates and their

biochemical reactions. More specifically, *Salmonella* isolates leading to monophasic *Arizona* serotypes joined subspecies IIIa, whilst all diphasic serotypes have been included into subspecies IIIb. Although these recommendations were not officially accepted by the International Committee on Systematic Bacteriology, many other organisations, e.g., the World Health Organization and the USA Centers for Disease Control and Prevention, have accepted them. In 1989, based on DNA relatedness studies, evidence was produced showing that *S. bongori* belonged in a distinct species (Reeves et al. 1989). In the subsequent years, several relevant papers have been published confirming or refuting the proposal of Le Minor and Popoff (1987) (e.g., Wayne 1994, Euzeby 1999, Ezaki, Amano et al. 2000, Ezaki, Kawamura and Yabuuchi 2000, Yabuuchi and Ezaki 2000). In 2005, the term '*enterica'* instead of '*choleraesuis'* has finally been accepted and a relevant decision has been issued ('Judicial Opinion 80') (Judicial Commission of the International Committee on Systematic Bacteriology 2005). Thus, *Salmonella enterica* became the type species of the genus, replacing *Salmonella cholerasuis* (Tindall et al. 2005).

Currently, both *Salmonella* spp. classification systems are in use. As per recent amendments made by the International Committee on Systematic Bacteriology, the genus *Salmonella* includes two species: *Salmonella enterica* and *Salmonella bongori.* The former includes six subspecies: 'arizonae', 'diarizonae', 'salamae', 'enterica', 'houtenae' and 'indica'. *Salmonella* isolates (subspecies I) are usually detected in warm-blooded animals, while salmonellae classified to the other subspecies usually originate from cold-blooded animals and the environment. The antigenic classification system counts today over 2,600 serotypes or serovars (Issenhuth-Jeanjean et al. 2014). Although both terms are frequently used, according to the Rules of Bacteriological Code (1990 Revision), the latter term is preferable and has been adopted by many institutions. All the different recognised formulae are registered in a list, known as Kauffman-White-Le Minor scheme, which is held by the WHO Collaborating Centre for Reference and Research of *Salmonella* at the Pasteur Institute (Nataro et al. 2011). This scheme is updated annually, with newly reported serotypes appropriately listed therein.

Serovars are further classified in biotypes and phagotypes. Biotyping takes under consideration the biochemical profile of isolates within the same serotype. In accordance, phagotyping assesses sensitivity of the isolates to lysis by bacteriophages among isolates of the same serotype.

Although all the above have contributed significantly in establishment of a widely accepted nomenclature system for the organism, the problem of the differing taxonomic interpretations among the researchers does not appear to have been solved. This fact indicates that neither system has

the capacity to provide clear limits among salmonellae. Consequently, the improvement efforts of the two classification systems are expected to be continued.

Nomenclature

Nomenclature of salmonellae is a complex issue. Establishment of serotyping has changed nomenclature of salmonellae and serovars began to be designated by their antigenic formula, adopting the one serovar - one species pattern. However, *Salmonella* spp. classification into subspecies has changed again the format of naming of the different isolates, adding subspecies determination prior of antigenic composition. Current nomenclature system includes both the Kauffman-White scheme and decisions of the International Committee on Systematic Bacteriology, in an attempt to unify the two systems and minimise differences.

Currently, salmonellae are designed with a complex sequence, which includes classification of each isolate and a short report of their antigenic formula. For many serovars, traditional names are still in use. Often, following subspecies name, bacterial antigens are reported. 'O' antigens are referenced first and defined with arabic numerals. Altogether, 67 different O factors have been recognised; among these, O antigens susceptible to mutation are indicated in brackets. 'H' antigens are referenced with small letters (phase 1 antigens) or arabic numerals or small letters (phase 2 antigens). To separate numbers of each specific antigen, quotemarks (' ') are used. When an isolate does not express 'O' or 'H' antigens, it is described with '-' in the relevant position.

The nomenclature system of salmonellae is summarised in Table I.i.

Table I.i. Current approach for nomenclature of *Salmonella* genus bacteria (Su and Chiu 2007, Ryan et al. 2017).

Genus[1]	Species[2]	Subspecies[3]	Serovars[4]	Number of serovars
Salmonella	*enterica*	*enterica* (or I)	Typhi, Paratyphi	1586
		salamae (or II)	9,46:z:z39	522
		arizonae (or IIIa)	43:z29:-	102
		diarizonae (or IIIb)	6,7:l,v:1,5,7	336
		houtenae (or IV)	21:m,t:-	76
		indica (or VI)	59:z36:-	13
	bongori		13,22:z39:-	22

1. capitalised, italics; 2. Italics; 3. Italics; 4. capitalised, non-italics.

B. BRIEF APPRAISAIL OF *SALMONELLA* SPP. INFECTIONS IN SHEEP

Introduction

Incidence risk of clinical salmonellosis in sheep remains low, not exceeding 1%, or rarely, 2%. Onset of clinical disease is usually triggered by stressors, e.g., transport, overcrowding, lambing, malnutrition. Concurrent diseases, e.g., heavy gastrointestinal nematode infection (Dipineto et al. 2012), may also activate a previously suppressed infection. Specifically *Salmonella enterica* subsp. *enterica* ser. Typhimurium may cause clinical conditions without any predisposing factors. Often, an infection leads in outbreaks of local extent, involving large number of animals, usually characterised by increased severity of clinical signs and morbidity within a flock; fatality rates in naïve flocks may reach up to 90%. Clinical signs of salmonellosis vary, depending on serovar involved and animal factors (e.g., age). In general, two major distinct syndromes may develop; one is manifested with profuse diarrhoea, consequently of acute enteritis, the second leading in abortion cases in ewes, usually those in the final 6 to 4 weeks of pregnancy.

Usually, *Salmonella* infections remain asymptomatic. Individual sheep can serve as active carriers of the pathogen for a variable period of time with no clinical signs. Moreover, such animals may often excrete *Salmonella* spp. intermittently for some time, thus contributing in maintaining and spreading the organism. Recent surveillance studies, based in *Salmonella* spp. detection in faecal samples, have suggested that approximately 2% of clinically healthy sheep may be carriers of the pathogen (Esmaeili and Kalateh 2016, Stipetic el al. 2016, Nouichi et al. 2018). Other studies, conducted in abattoirs, have shown that prevalence of *Salmonella* spp. infections in sheep carcasses ranged between 0.5% to 12% (Woldemariam et al. 2005, Molla et al. 2006, Oloya et al. 2007, Stipetic et al. 2016).

Clinical conditions of the gastrointestinal tract

Acute enteritis is the most commonly occurring clinical syndrome caused by *Salmonella* in sheep, affecting both adult and young animals. The syndrome is characterised by watery, green-coloured to blood-tinted diarrhoea, which can lead in severe dehydration, disturbance of acid-base

balance, endotoxaemia and death. Fever (> 41°C), reluctance to move, depression, dullness, loss of appetite and reduced milk produced may also be evident in early stages of the disease. Occasionally, animals may be found dead, prior of showing any clinical signs.

In newborn or young lambs, several *Salmonella* serovars can cause systemic septicaemia. Septicaemic syndrome is most frequently caused by *S.* Typhimurium and, usually, affected lambs die within 12 hours from the onset of disease. In untreated cases, fatality rate of an outbreak may reach 75%, while recovery of diseased animals may take up to two months, causing significant growth retardation. Molecular studies have revealed that the organism persists in lymph nodes or tonsils, even when no *Salmonella* could be detected in faeces. Under stress, recurrent episodes of diarrhoea may occur. Besides acute enteric salmonellosis, subacute or chronic forms of disease have also been recognised (Richards et al. 1993). In such cases, it may be difficult to determine whether *Salmonella* spp. is the principal cause of the disease.

Hitherto, several *Salmonella* serovars have been described to cause outbreaks of diarrhoea in adult sheep (Table I.ii). Although systemic monitoring of salmonellosis in sheep is not routinely performed, *S*. Typhimurium is the most commonly reported *Salmonella* serovar (Sojka et al. 1983, Davies et al. 2004). *S.* Typhimurium usually causes severe necrotic enteritis in the ileum and large intestines, leading in increased morbidity and mortality rates (Hunter et al. 1976). The organisms is excreted from infected animals only for a short period; animals that survive the septicaemic stage do not tend to become carriers. Other serovars that have been isolated from cases of diarrhoea and deaths in both adults and lambs are shown in Table I.ii.

Table I.ii. Infections reported in sheep, caused by various serovars of *S. enterica* subsp. *enterica*.

S. enterica subsp. *enterica* serovar	Clinical signs	Reference
Anatum		Kumar et al. (1972), Woldemariam et al. (2005)
Bovismorbificans	diarrhoea	Richards et al. (1989)
Derby	abortions	Findlay (1973)
Enteritidis		Atyabi et al. (2012)
Heidelberg		Kalchayanand et al. (2007)
Indiana	abortions	Luque et al. (2009)
Kentucky		HM Government 2018
Newport		HM Government 2018
Oranienburg	abortions	Russell and Tannock (1964), Hazlett et al. (2013)

Abortions

Abortions can occur in cases of *Salmonella* spp. infection in pregnant ewes. In some countries (e.g., Cyprus, Spain, France, Croatia, New Zeeland) this is the main, if not the only, clinical condition associated with salmonellosis in sheep.

Clinical signs vary according to the serovar. In some cases, severity of the disease can lead in death of affected ewes. In others, infected ewes may not abort, but deliver stillbirth or weak lambs, which often may die within two weeks of birth.

Salmonella enterica subsp. *enterica* ser. Abortusovis and *Salmonella enterica* subsp. *enterica* ser. Brandenburg are the two most commonly isolated abortifacient serovars (Wray and Linklater 2000, Kerslake and Perkins 2006, Masala et al. 2007).

S. Abortusovis is a sheep-restricted serovar. The pathogen tends to present an endemic pattern in the frequency of abortions, which are also reported as '*paratyphoid abortion'* (Vickers et al. 1958). Incidence risk of the infection varies from 1.7% to 10.5% of abortions cases in ewes (Wirz-Dittus et al. 2010). In Greece, a field study has revealed mean prevalence of seropositivity in flocks to be 24.5% (Giannati-Stefanou et al. 1997). After infection, abortion may take place in 30 to 50% of ewes in a flock, reaching up to 90% in flocks affected for the first time (Jack 1968, Wray and Linklater 2000). Ewes in their first pregnancy appear to be more susceptible. The organism is excreted in faeces for up to three months and in vaginal secretions for as long as one year after abortion (Belloy et al. 2009). The organism has also been recovered from colostrum and milk samples of ewes that had aborted, as well as, rarely, in samples of preputial fluid of rams in infected flocks (Sanchis et al. 1986).

S. Brandenburg is a major cause of abortions in ewes in New Zealand. Most outbreaks occur during the autumn or spring, with abortions manifested within a period of two to three weeks. Multiparous ewes appear to be more susceptible to infection and more likely to experience an abortion. Affected ewes may develop moderate diarrhoea prior of abortion (Bailey 1997). Sudden death of apparently healthy ewes has been also reported. In previously uninfected flocks, abortion rate ranged between 3% to 20%. Mortality rate in ewes that had aborted, ranged between 40 to 50%, although death of all infected ewes in a flock has also been reported (Bailey 1997, Clark et al. 2004). In subsequent years, both morbidity and mortality rates decrease gradually. The organism is excreted in large numbers in aborted foetuses and uterine discharges of ewes. *S.* Brandenburg is excreted in the faeces of affected ewes for up to 6 months after abortion; it remains infective in the environment for up to 3 months (Robinson 1967).

Salmonella enterica subsp. *enterica* ser. Montevideo, *S.* Dublin and *S.* Typhimurium are also notable causes of abortion in sheep (Linklater 1983, Sharp et al.1983, Uzzau et al. 2000). About 10% of ewes affected by *S*. Montevideo may become ill after abortion. It should be also mentioned that in flocks infected by *S.* Typhimurium or *S*. Dublin, abortions may frequently co-exist with cases of diarrhoea in lambs and, possibly, signs of illness in other animals on farm.

Other clinical conditions

In lambs, symptoms of pneumonia or polyarthritis caused by *Salmonella* spp. have been also reported. Apart from S. Abortusovis, various other serovars (*S.* Montevideo, *S.* Dublin, *S.* Indiana) have also been incriminated in cases of abortions in ewes (Baker et al. 1971, Linklater 1983, Luque et al. 2009).

C. CLINICAL CONDITIONS IN ANIMALS ASSOCIATED WITH *SALMONELLA ENTERICA* SUBSP. *DIARIZONAE*

Characteristics of *Salmonella enterica* subsp. *diarizonae*

Taxonomic considerations

Salmonella isolates, currently named *Salmonella enterica* subsp. *diarizonae*, have been first detected in faecal samples from reptiles (Caldwell and Ryerson 1939). They were classified into the *Salmonella* 'Arizona' group, subsequently renamed as subgenera Arizona or subgenus III. In 1987, subgenus III was further divided into *S. enterica* subsp. *arizonae* and *S. enterica* subsp. *diarizonae* (also known as subsp. IIIa and IIIb, respectively). The discrimination was performed based on their differing reactions in biochemical tests and their genomic relatedness (Tindall et al. 2005). The most remarkable divergence between these two subspecies was that all serovars belonging to *diarizonae* subspecies were diphasic for the H antigen. Nowadays, at least 336 distinct serovars of *S. enterica* subsp. *diarizonae* have been detected, accounting for approximately 13% of all recorded serovars within the *S. enterica* species (Grimont and Weill 2007, Lamas et al. 2018).

S. enterica subsp. *diarizonae* isolates are most frequently recovered from samples collected from cold-blooded animals or the environment. In addition, they may also be naturally harboured by domestic or wild warm-blooded animals. Humans may also be infected. As with all salmonellae, *S. enterica* subsp. *diarizonae* mainly colonises the gastrointestinal tract of hosts, more specifically the anterior part of the small intestine. Nevertheless, extra-intestinal infections have been also reported in sheep and humans. The vast majority of infections associated with *S. enterica* subsp. *diarizonae* remains un- or mis-diagnosed, as they are accompanied with mild and self-limited clinical signs. Severe disease rarely develop, mainly affecting young, elderly or/and immunocomprised individuals.

Identification matters

The biochemical characteristics of *S. enterica* subsp. *diarizonae* differ a lot from other enterica subspieces; in most cases, these are outside from the general principles described for salmonellae. Most *S. enterica* subsp. *diarizonae* isolates are lactose-fermenters (*lac*+); it has been estimated that almost 85% of *S. enterica* subsp. *diarizonae* isolates were lactose-fermenters: either

lactose-fermenters (producing both β-galactosidase and permease) or delayed lactose-fermenters (producing only β-galactosidase) (Goodman and Pickett 1966, Davies et al. 2001). It is noteworthy that, in contrast to the above, within all other *S. enterica* subspecies only 1% of isolates, cumulatively, are lactose-fermenters (Ewing 1986, Popoff and Le Minor 2005). Recent genome sequencing studies have shown that lactose fermentation in *S. enterica* subsp. *diarizonae* is mainly associated with presence of extended *lac* operon (also known as lac region), more specifically with a functional *lac* gene (Barrow and Methner 2013). It is noteworthy that, according to Leonard et al. (2015), the *lac* gene of *S. enterica* subsp. *diarizonae* isolates is relatively conserved and shares low sequencing homology (less than 73%) with the other *enterica* or non-*enterica* subspieces. Beyond lactose utilisation, the majority of *S. enterica* subsp. *diarizonae* isolates ferment citrate and D-sorbitol and can use malonate and lactose as sources of carbon ('malonate-' and 'β-galactosidase-positive'). In contrast, they do not ferment dulcitol and they lack the tryptophanase enzyme, giving a negative result in the conventional indole test ('indole-negative').

The basic cultural requirements of *S. enterica* subsp. *diarizonae* are similar to those of other salmonellae. However, bacterial identification and serotyping can be difficult. Unlike other *Salmonella* serovars, *S. enterica* subsp. *diarizonae* is usually found in small numbers in faecal samples, even from individuals with clinical disease. As the result of bacterial competition in the gastrointestinal tract, often, growth of *S. enterica* subsp. *diarizonae* can be limited *in vivo*, which may be followed by further competition during culturing, this ultimately possibly leading in limiting or suppressing development of colonies of the organism in culture media (Alvseike and Skjerve 2000). Further, the organism is intermittently excreted in faeces, which can also reduce chances of recovery from relevant samples (Alvseike et al. 2004). Therefore, the pre-enrichment and enrichment steps (37 ^{o}C for 16 - 18 h) have a significant role in the identification process of these bacteria (Bonke et al. 2012).

Due to the wide variety of results of biochemical reactions, discrimination of *S. enterica* subsp. *diarizonae* from other Enterobacteriaceae by use of conventional microbiological techniques can be difficult. Often, application of time-consuming and expensive techniques may be required (Arrayo and Arrayo 1995, Coleman et al. 1995). In fact, the ability of *S. enterica* subsp. *diarizonae* to ferment lactose poses an obstacle to its optimal isolation and identification. The most commonly used media for *Salmonella* detection have been developed on the assumption of non-lactose fermentation, hence they are inappropriate for differentiation and presumptive identification of this organism (McDonough et al. 2000, Andrews et al. 2018). Moreover, even using appropriate selective and differential media, morphology of *S. enterica* subsp. *diarizonae* colonies provides limited information. Typical morphology colonies on those media show similar appearance to colonies of

other Enterobacteriaceae. Moreover, atypical colonies of *S. enterica* subsp. *diarizonae* have also been reported (Soren et al. 2015), having an unusual appearance in particular media (Alvseike and Skjerve 2000). One should mention *Citrobacter freundi*, an organism that produces colonies very similar to *S. enterica* subsp. *diarizonae* and also has an identical biochemical pattern to that organism (Walt and Steyn 1989), that way potentially leading to misidentifications. The results of the various biochemical tests that can be applied for identification of the organism are summarised in Table I.iii (Ewing 1986).

Table I.iii. Summary of results of biochemical tests that can be performed for identification of *S. enterica* subsp. *diarizonae* (Ewing 1986).

Biochemical reaction	Result	Biochemical reaction	Result	Biochemical reaction	Result
Methyl red test	+	Voges-Proskauer test	-	Lipase test	-
Lysine decarboxylase test	+	Malonate utilisation test	+	Acetone utilisation test	(+)
Tartrate utilisation	(-)	Galacturonate metabolism	+	Citrate reduction	+
ONPG reaction	+	Nitrate reductase activity	+	KCN tolerance	-
Gelatine liquidifation	-	Aesculine hydrolysis	-		
H_2S production	+	Indole production	-	Catalase production	+
D-glucose acid production	+	Urease production	-	Cytochrome C oxidase production	+
D-glucose gas production	+				
D-sorbitol fermentation	+	Ornithine fermentation	+	Salicin fermentation	-
Lactose fermentation	(+)	Glycerol fermentation	-	Adonitol fermentation	-
Cellobiose fermentation	-	D-mannose fermentation	+	Sucrose fermentation	-
Dulcitol fermentation	-	Maltose fermentation	+	Raffinose fermentation	-
L-rhamnose fermentation	+	L-arabinose fermentation	+	myo-Inositol fermentation	-
Melibiose fermentation	+	Trehalose fermentation	+		

+: positive result, (+): positive results in > 70% of isolates, (-): negative result in > 70% of isolates, -: negative result.

Use of novel technologies has allowed a deeper knowledge on the organism. Based on phylogenetic studies, *S. enterica* subsp. *diarizonae* has been classified between *S. houtenae* and *S. arizonae* (Porwollik et al. 2002, Fookes et al. 2011, Desai et al. 2013). Genome sequence studies of the organism have revealed remarkable divergences between *S. enterica* subsp. *diarizonae* and other *enterica* or non-*enterica* subspecies of *Salmonella*. In many cases, those divergences have been connected with certain characteristics of the organism. The most significant genomic differences have been found to be located to SPI-1 and SPI-2, where two major virulence factors (secretion systems T3SSs and T4SSs, respectively) are encoded (Hensel 2004, Byndloss et al. 2017). Genomic losses and local mutations, which had been done at these '*Salmonella* pathogenicity islands', have been considered to be responsible for the limited invasive properties of *S. enterica*

subsp. *diarizonae*. SPI-6 presents a notable differentiation from the other serovars: these isolates show lack of VI secretion system (T6SS), which is an essential factor able to compress gut bacterial competition and establish an infection within the host intestine (Desai et al. 2013). A similar to T6SS virulence factor is encoded in isolates from SPI-21, but it has little or no homology with those encoded to SPI-6. This specific T6SS, encodes a number of proteins (pyocins), which are used by the organism to kill competitive bacteria (Hachani et. al. 2014).

S. enterica subsp. *diarizonae* serovar 61:k:1,5,(7) (also known as serovar *diarizonae*) is often isolated from samples from sheep, often prevailing over all other *Salmonella* subspecies or serovars. Isolates of the serovar have also been recovered from other farm animals (pigs, poultry, cattle), as well as from several wild mammals. Despite its high occurrence, clinical conditions associated with this serovar have been rarely reported. Currently, it is considered to be of low pathogenicity and possibly a host-adapted *Salmonella* serovar. The majority of these isolates are considered to be late-lactose fermenters (Long et al. 1978).

Virulence aspects

There are few reports regarding behaviour of the organism in the gastrointestinal tract. These refer to findings during experimental infections with *S. enterica* subsp. *diarizonae* (Madsen et al. 1998, Pasmans et al. 2005, Katribe et al. 2009). Madsen et al. (1998) have suggested that the organism had limited invasive properties, similar to those of non-invasive *Salmonella* serovars.Thereafter, Pasmans et al. (2005) have described a decreased adhesion capacity for the organism. Finally, Katribe et al. (2009) have proposed a theory of defective colonisation for *S. enterica* subsp. *diarizonae* isolates; according to that, whilst isolates could colonise the intestine of the host for a long period (ever for a period longer than one year; Lacasta et al. 2017), they were unable to crossover intestinal barrier and potentially disseminate to neighbouring organs, as other serovars (e.g., *S.* Typhimurium) could do. Factors potentially accounting for the reduced invasiveness of these organisms would be as follows.

- Limited interaction with intestinal epithelial cells.
- Lack of production of effective adhesins.
- Poor replication within macrophages.

In view of the above, it is considered that extra-intestinal infections of *S. enterica* subsp. *diarizonae* might be associated primarily with immunocompromised hosts.

The *lac* operon of *S. enterica* subsp. *diarizonae* isolates may also play an important role for defective colonisation, causing weak bacterial invasion to epithelial cells. Jiang et al. (2015) have

inoculated a *lac*+ and a *lac*- isolate of *S.* Typhimurium to mice; mice challenged with the *lac*+ isolate survived, while those challenged with the *lac*- isolate died after challenge; genomic sequencing analysis has been revealed that *lac* operon of the *lac*+ isolate was capable to inhibit or modify expression of adjacent genes, e.g., those regulating flagellar biosynthesis, that way leading in reduced pathogenicity of the *lac*+ isolate. A similar mechanism may be valid for all *S. enterica* subsp. *diarizonae* isolates, as these are *lac*+ organisms.

The ability of the organism to form biofilm is also an important feature. Biofilm producing isolates of the organism have been isolated from poultry by Lamas et al. (2016a). These isolates have been shown to form biofilm at various temperatures and many different conditions (from 6 ^{o}C to 37 ^{o}C and after 48 h to 7 day incubation). The biofilm-associated gene *csgD* has been detected in those isolates.

Data regarding antimicrobial resistance of *S. enterica* subsp. *diarizonae* are limited. Possibly, this is the consequence of the isolation of the organism primarily from wild animals and infrequently from domestic animals, as well as its rather reduced pathogenicity in farm animals. Nevertheless, Mejia et al. (2006) have reported the isolation of a multi-resistant isolate from pigs, whilst Romero et al. (2016) have reported reduced susceptibility to streptomycin by isolates from captured reptiles. Antimicrobial susceptibility of serovar *diarizonae* isolates has been reported by Bonke et al. (2012), who described only mild resistance to colistin, and by Dargatz et al. (2015), who described negligible resistance to various antimicrobial agents.

Occurrence and clinical conditions

Occurrence in sheep

Clinical conditions

Salmonella enterica subsp. *diarizonae* is considered to be the most frequently detected salmonella subspecies in sheep, but, paradoxically, it has been little-studied. Most *S. enterica* subsp. *diarizonae* infections in sheep are caused from isolates with antigenic formula 61:k:1,5,(7), historically also known as ‘Salmonella arizona paracolon’ or else ‘Salmonella Arizona 26:29:30’ (Hall and Rowe 1980). Serovars with minor modifications or with incomplete antigenic structure are also frequent causal agents of disease. Many researchers believe that this specific serovar of this diarizonae subspecies is a commensal resident of sheep’s intestinal tract. The wide spread of the

serovar in sheep, the long shedding period and the presence of the organism in tissue samples from clinically healthy sheep provide adequate support to this hypothesis. In contrast, the prevalaing scientific opinion suggests that, whilst *S. enterica* subsp. *diarizonae* is a typical example of a host-adapted salmonella serovar, the organism retains its pathogenic properties and under certain conditions may cause significant intestinal and/or extra-intestinal clinical conditions.

In the early stages of *S. enterica* subsp. *diarizonae* research, the organism had been considered as a causal agent for abortion in ewes (Ryff and Browne 1952, Bruner et al. 1955, Harvey et al. 1966, Greenfield et al. 1973). Long et al. (1978) have reported presence of *S. enterica* subsp. *diarizonae* in samples from aborted or stillborn foetuses. The organism has also been recovered from flocks with cases of abortion (Hall and Rowe 1980, Sojka et al. 1983). However, in all cases, the organism had been isolated with various other pathogens, which made its potential role as causal agent of abortion unclear.

Oral challenge of pregnant ewes with the organism has not resulted in pathological results, with all ewes producing apparently healthy lambs (Hannam et al. 1986). Although the organism had been excreted from ewes for a long time, no clinical signs had been observed. The authors have concluded that the role of the pathogen (if any) would be of minor importance.

S. enterica subsp. *diarizonae* has also been recovered from faecal samples of lambs with diarrhoea. In two studies (Harp et al. 1981, Hannam et al. 1986), young lambs, orally challenged with the organism, were examined regularly thereafter. The organism was isolated from faecal samples after inoculation for a period of 6 to 8 days, although all lambs bar one remained clinically healthy; that one lamb, previously diagnosed with low serum globulin concentration, developed severe diarrhoea and died within 48 hours post-inoculation.

The most common extra-intestinal infection by *S. enterica* subsp. *diarizonae* in sheep occurs in the respiratory system, specifically in the upper respiratory tract. Incidents of chronic proliferative rhinitis caused by the organism have been reported in adult sheep by Meehan et al. (1992) and Brogden et al. (1994). Further cases of the pathological conditions caused *S. enterica* subsp. *diarizonae* ser. 61:k:1,5,(7) have been described by Lacasta et al. (2012), who presented details of the problem from 10 adult sheep with moderate respiratory clinical signs; at post-mortem examination, extensive nasal obstruction caused from proliferative tissue, has been detected. More clinical cases have been reported by Stokar-Regenscheit et al. (2017) with features similar to those described by Lacasta et al. (2012). Experimental studies, in which the same serovar had been used (Lacasta et al. 2017), have shown the presence of the organism in the nasal chambers, as well as in faecal samples.

Finally, a case of suppurative orchitis and epididymitis caused by the organism has been reported by Ferreras et al. (2007). The animal was presented with signs of acute unilateral orchitis (pain during palpation of the scrotum and scotral enlargement), whilst reduced fertility was also evident.

Prevalence of the infection

In Europe and North America, isolates of *S. enterica* subsp. *diarizonae* ser. 61:k:1,5,(7) belonging to this specific serovar are frequently recovered from sheep (Lamas et al. 2018). In contrast, no reports of isolation of the organism have been reported from Australia and South Africa.

In the United Kingdom, Sojka el. (1983) were the first to report recovery of such isolates from sheep. Later, a retrospective study by Davies et al. (2001) has indicated that frequency of *S. enterica* subsp. *diarizonae* ser. 61:k:1,5,(7) detection in clinical samples from sheep was increasing rapidly: in 1998 the organism was the predominant *Salmonella* serovar recovered from sheep and in 1999 the relevant infections accounted for 46% of all *Salmonella* serovars detected. In an abattoir survey, Milnes et al. (2008) recorded 0.6% isolation rate of *S. enterica* subsp. *diarizonae* ser. 61:k:1,5,(7) infections from carcasses, which accounted from 60% of all *Salmonella* isolates detected.

In Norway, many studies have shown an isolation rate of the organism varying from 1% to 21%. (Alvseike and Skjerve 2002, Sandbergab et al. 2002). Moreover, in abattoir surveys, it was found that the organism was isolated from 12% of sheep flocks in the country. In neighbouring Sweden, isolation of the organism from sheep flocks has been reported from 18% of sheep flocks whilst isolation rate from carcasses was 0.5% (Soren et al. 2015).

In Switzerland, in a study with a different approach, in which molecular methods have been employed for detection of *S. enterica* subsp. *diarizonae* ser. 61:k:1,5,(7), detection rate in tissue samples collected from slaughterhouses was 43% (Bonke et al. 2012)

Occurrence in other warm-blooded animals

S. enterica subsp. *diarizonae* in other warm-blooded animals (i.e., apart from sheep) has been sporadically reported. In general, prevalence of infections is very low (van Duijkeren et al. 2002, Davies et al. 2004).

Prevalence of infection in cattle in Sweden has been found to be < 0.1% (Swedish Veterinary Association 2014). Isolation of the organism from pigs has been reported once from one sample in Greece (Evaggelopoulou et al. 2018). The organism has also been isolated once from a domestic

cat (Swedish Veterinary Association 2012). Whilst there is a large number of relevant studies performed in poultry, only one isolation of *S. enterica* subsp. *diarizonae* has been reported from 4,600 samples collected over a period of five years in the United Kingdom (HM Government 2018) and isolation rate in Spain was found to be < 0.02% (Lamas et al. 2016b).

The organism has also been recovered from samples from several warm-blooded wildlife species (Table I.iv). Wild boars and wild birds have been found to be infected frequently: prevalence has been reported to be as high as 13.0% (Chiari et al. 2013); in fact, in those animals *S. enterica* subsp. *diarizonae* is the second most frequently identified salmonella (Chiari et al. 2013, Zottola et al. 2013). Further, the organism has been sporadically isolated from samples collected from other wildlfe animals: red foxes, deer, moose, coyotes, white wagtail and sparrows. Details are in Table I.iv.

Table I.iv. Summary of reported isolations of *S. enterica* susp. *diarizonae* from wildlife animals.

Country where isolation took place	Animal species, from which recovered	*S. enterica* susp. *diarizonae* serovar	Reference
Italy	Wild boar	50:r:1,5,7 61:c,z35 61:i:e,n,x,z15	Zottola et al. 2013
	Red fox	17:z10:e,n,x,z15 (not provided)	Chiari et al. 2014
Norway	Red fox	61:k:1,5,(7)	Handeland et al. 2008
	Deer	(not provided)	Jørgensen et al. 2016
Spain	Wild boar	38:IV:z35	Millan et al. 2004
	White wagtail House sparow	61:-:1,5,7 61:-:1,5,7	Andres at al. 2016
Sweden	Cat	(not provided)	Swedish Veterinary Association 2012
	Red fox	50:z52:-	Swedish Veterinary Association 2013
	Moose	38:r:z35	
	Moose	(not provided)	Swedish Veterinary Association 2015
Mexico	Coyote	48:i:z 50:r:z	Jay-Russell et al. 2014

Occurrence in cold-blooded animals

S. enterica subsp. *diarizonae* is considered to be part of the normal intestinal microbiota of cold-blooded animals, including marine organisms.

Lacertilians and amphibians are susceptible to *S. enterica* subsp. *diarizonae* infections. It has been documented that these species serve as asymptomatic carriers of the organism (Onderka and

Finlayson 1985, Monzon et al. 1995, Mitchell and Shane 2000, Ebani et al. 2005, Pasmans et al. 2005, Bauwens et al. 2006, Pedersen et al. 2009). Often, over one serovar and/or isolates may be recovered from the same animal. The organism colonises the gastrointestinal tract of hosts and can be excreted leading to its dissemination. However, details of the potential pathogenicity of the organism remain unknown (Tellez et al. 2002), although it is generally assumed that *S. enterica* subsp. *diarizonae* may cause disease of varying severity in immunocomprised or stressed animals.

Spontaneous isolation of the organism from various cold-blooded animals has been reported. In snakes, prevalence varied from 2.0% to 6.5% in wild individuals and up to 54.0% in captive ones (Geue and Loschner 2002, Lukac et al. 2015, Romero et al. 2016). In lizards, prevalence varied from 3.5% to 18.0% (Geue and Loschner 2002, Pasmans et al. 2005, Wikström et al. 2014). In turtles, prevalence was found to be 1.5% (Marin et al. 2013). Finally, the organism has also been isolated from frogs and toads (Ribas and Poonlaphdecha 2017).

Occurrence in humans

S. enterica subsp. *diarizonae* infections and the respective clinical conditions in humans have been reported since the 1950s (Saphra and Wassermann 1954, Saphra and Winter 1957, Laylee 1957). Prevalence of infection is low (Aleksic et al. 1996) compared with other salmonella subspecies and serovars and clinical cases caused bu this organism are only sporadically reporded. In Europe, most *S. enterica* subsp. *diarizonae* isolations from human samples are recorded in France (European Centre for Disease Prevention and Control 2018). In Greece, since 2011, 17 isolates of *S. enterica* subsp. *diarizonae* have been recovered from samples of human origin, although the serovars of these isolates have not been identified (Mellou K 2018, personal communication).

Infection processes in humans seem to be similar to those in other mammals (Abbott et al. 2012). The organism has been recovered from samples from blood, central nervous system, heart, lungs, gastroinstestinal tract, spleen, urine and bones. Children younger than 5 years and immunocompromised and elderly individuals are considered to develop a more severe disease after infection by *S. enterica* subsp. *diarizonae* than healthy adults (Chong et al. 1991, Hervas et al. 2012, Abbot et al. 2012, Eke et al. 2014, Bhatia et al. 2015, Horvath et al. 2016). Thusfar, no cases of clinical disease caused specifically by *S. enterica* subsp. *diarizonae* ser. 61:k:1,5,(7) have been reported in the literature.

Zoonotic implications of infections in animals

Animal to human transmission of *S. enterica* subsp. *diarizonae* has been repeatedly reported. People may be infected either by direct contact with infected animals or by consumption of raw or inappropriately cooked food or contaminated water (Madsen et al. 1998, Tavechio et al. 2002, Arslan and Eyi 2010, Hoelzer et al. 2011, Strawn et al. 2013, Reddy et al. 2016, Heredia et al. 2018). Farm and other domestic animals seemingly have a limited role in the transmission of the pathogen to humans. Cold-blooded animals, as important reservoirs of the organism, have been implicated in many cases of animal to human transmission, apparently as the result of direct or indirect contact. During the period 2005 to 2012, in Europe, nine cases or transmission of *S. enterica* subsp. *diarizonae* (various serovars, but not including 61:k:1,5,(7)) from snakes or lizards to people have been reported in the literature (Bruins et al. 2006, Bertrand et al. 2008, O'Byrne et al. 2008, Hervas et al. 2012).

Nevertheless, the zoonotic potential of the specific serovar 61:k:1,5,(7) is still a matter of debate. So far, there has been no report of any proven case of zoonotic transmission of that organism to people. Even in regions with increased prevalence of *S. enterica* subsp. *diarizonae* 61:k:1,5,(7) infections in sheep, respective prevalence of infections in humans is low and similar to that in areas with low prevalence of animal infection. Further, people working with sheep (e.g., farmers, abattoir employees) do not show particularly increased infection rates, despite their increased exposure to the organism.

It is noteworthy than in Sweden carcasses and meat products from animals infected with *S. enterica* susp. *diarizonae* 61:k:1,5,(7) are not condemned, but can be given for human consumption with no limitations (Sörén et al. 2015), this being in sharp contrast to relevant legislation for other *Salmonella* species and serovars.

CHAPTER II

FIELD INVESTIGATIONS INTO THE IMPLICATION OF *SALMONELLA ENTERICA* SUBSP. *DIARIZONAE* IN THE DIARRHOEIC SYNDROME OF LAMBS

A. INTRODUCTION

Available data regarding the potential role of *Salmonella enterica* subsp. *diarizonae* as a cause of the diarrhoeic syndrome of lambs are limited. In the past, there have been some reports regarding detection of the organism from lambs with diarrhoea (Harp et al. 1981, Hannam et al. 1986). These cases referred to sporadic incidents in individual lambs, with within-affected farm investigations, rather than wider investigations of the problem. There is little evidence available regarding the presence of the organism in sheep farms

Objective of this work was the study of the extent of infection of lambs by *S. enterica* subsp. *diarizonae* in sheep farms with clinically evident diarrhoeic syndrome in lambs and the serotyping of isolates of the organism isolated from cases of the disease. In this chapter, a case of diarrhoeic syndrome in lambs in a farm, caused *Salmonella enterica* subsp. *diarizonae*, is described. Then, the results of an extended field work regarding the presence of *S. enterica* susp. *diarizonae* in diarrhoeic lambs of region of Thessaly are presented.

B. CASE DESCRIPTION: ISOLATION OF *S. ENTERICA* SUBSP. *DIARIZONAE* FROM LAMBS DURING AN OUTBREAK OF DIARRHOEIC SYNDROME IN A FARM

Introduction – Farm visit – History

In October 2014, an investigation started in a sheep farm located in Mitropolis, Karditsa, Greece (coordinates: 39.325 N, 21.820 E). The farm was managed according to the semi-intensive system. The farm had been under monitoring at the time, as during the preceding month (September 2014) cases of bluetongue had been diagnosed in sheep therein, after the outbreak of the disease had started in Greece (Vasileiou et al. 2016).

Two weeks after start of the lambing season, frequent cases of diarrhoea in young (5 - 15 day old) lambs were reported by the farmer, within a period of 7 days. Despite administration of first-line antimicrobial agents by the farmer, cases had continued. The farm was visited and a detailed investigation into the problem was initiated.

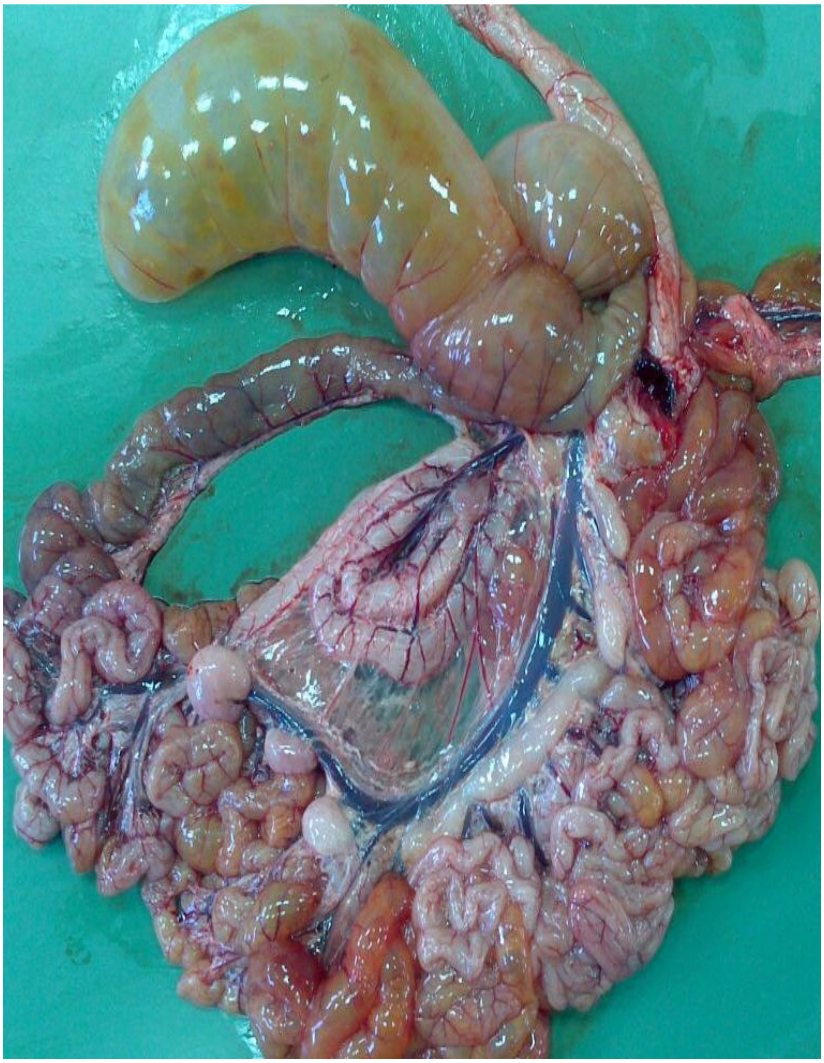
In total, 101 ewes had lambed by the time the investigation started and had produced 160 lambs. Of these lambs, 25 had been ill with diarrhoea (incidence rate: 0.156). Among sick lambs, 10 had already died when the investigation started (case fatality: 0.400). The farmer reported that animals developed diarrhoea and loss of appetite. Deaths occurred within 12 hours post-appearance of diarrhoea.

Clinical and post-mortem examination – Sample collection

Detailed clinical examination was performed in eight lambs with clinical signs available at the time of the visit to the farm. Animals were found dehydrated and weak. Rectal temperature was within the normal range for lambs (< 41.0 ^{o}C). No abnormal findings were recorded in heart or respiratory rates. Diarrhoea was yellow-coloured and watery.

A detailed post-mortem examination was performed in two lambs that had died before start of the investigation and were available. Salient findings were: empty gastrointestinal tract, clear evidence of inflammation in jejunum and ileum in the small intestine and presence of petechiae in the intestinal wall, which was thinner than normal (Figure II.1).

Figure II.1. Digestive tract during post-mortem examination of a lamb that had died within 12 hours after occurrence of diarrhoea.



From live animals, faecal samples were collected directly from the rectum, by following standard procedures, as follows: (a) by means of a sterile swab and (b) through insertion of a gloved finger of the investigator. Samples were collected from lambs with clinical signs (n = 7), which had started the earliest 48 hours prior to sampling. During post-mortem examination, samples of small intestine, liver and gallbladder were collected by using the aseptic technique from all lambs in which post-mortem examination had been performed (n = 2). Samples were stored into portable refrigerators with ice packs and transported to the laboratory.

Bacteriological, parasitological and virological tests in samples collected during initial farm visit

Materials and methods

Swab samples were plated onto 5% sheep blood agar plates for aerobic and microaerophilic incubation and McConkey plates for aerobic incubation at 37 ^{o}C for up to 48 hours. Colonies grown were observed for morphology and then processed for identification by conventional techniques (Barrow and Feltham 1993). Bacterial identification was performed by using the API rapid identification system (Biomerieux, Marcy-l'-Etoile, France).

Further, a part of faecal samples was initially examined by means of direct microscopy and processed by means of the modified Ziehl-Neelsen. Another part of faecal samples was examined by means of rapid commercial immunochromotography tests for detection of *Coronavirus* and *Rotavirus* (Canine Combined Rapid Test Kit CPV+CCV+CRV Ag; Quicking Biotech, Pudong New Area, China) and of *Adenovirus* and *Rotavirus* (Vikia Rota-Adeno; BioMerieux, Marcy-l'-Etoile, France).

Results

The bacteriological tests revealed abundant growth of *Salmonella enterica* in pure culture from two samples from each of the two dead lambs, specifically from the small intestine and the liver, and from four samples from live animals with clinical signs. The parasitological and virological tests did not yield positive results.

Subsequent laboratory tests: detailed identification of the isolated bacteria and antimicrobial agents susceptibility testing

Materials and methods

Initially, the automated identification Vitek 2 system (Biomerieux) was employed for confirmation of identification of all *Salmonella* isolates obtained as above.

Subsequently, the disk diffusion test (Kirby-Bauer antibiotic test) was employed to evaluate susbeptibility of all recovered *Salmonella* isolates to various antimicrobial agents. Disks (Sensi-Disks; Becton Dickinson, Franklin Lakes, USA) containing ampicillin (10 μg), cefquinome (30 μg), enrofloxacin (5 μg), erythromycin (2 μg), gentamicin (10 μg), marbofloxacin (5 μg) and trimpethoprim plus sulfadizine (5 μg). A disk without antibiotic was added on each Petri dish as negative control indicator; a Petri dish culture with *Staphylococcus aureus* strain NCTC6571 (National Collection of Type Cultures, London, United Kingdom) as the control organism, was also used. Test and control organisms were prerared identically and examined simultaneously. Colonies from a blood-agar plate were suspended in 2 mL sterile saline to a density approximately equal to MacFarlands Opacity Standard No. 1. A dry cotton wool swab was immersed into the suspension and excess liquid was drained by expressing it against the inside of the bottle. The bacterial suspension was inoculated onto Mueller-Hinton agar (Thermo Fisher Scientific, Waltham, USA) with the swab in such a way that the whole surface of the agar was covered. Then, disks were applied. Plates were incubated aerobically for 24 hours at 37 ^{o}C and subsequently the results were recorded as susceptible, intermediate or resistant by measuring the inhibition zone diametre (Garrod et al. 1981), according to the interpretive standards of the European Committee on Antimicrobial Susceptibility Testing (2014) and by comparing the inhibition zone sizes to those of the control organism.

Finally, one isolate was subcultured and sent to the Greek National Reference Laboratory for Salmonellae (in animals), which is a service of the Greek Ministry of Rural Development and Food, for serotyping. Another isolate was sent to the Spanish Technological Centre I+D for Food Safety also for serotyping.

Results

Results of testing in Vitek 2 confirmed that the isolates were *S. enterica*. Results of the biochemical tests were the same in all isolates (Table II.i). Results of susceptibility to antimicrobial agents were also the same for all isolates (Table II.ii).

Table II.i. Results of biochemical tests obtained in Vitek-2 system during testing of *Salmonella* isolates.

Biochemical reaction	Result	Biochemical reaction	Result
Adonitol	-	Glu-Gly-Arg-Arylamidase	-
Ala-Phe-Arylamadise	-	Glutamyl Arylamidase pNA	-
Alpha-Galactosidase	+	Glysine Arylamidase	-
Alpha-Glucosidase	-	H_2S Production	+
Beta-Alanine Arylamidase pNa	-	L-Arabitol	-
Beta-Galactosidase	+	L-Histidine assimilation	-
Beta-Glucosidase	-	L-Lactate alkalinisation	-
Beta-Glucoronidase	+	L-Lactate assimilatiion	-
Beta-N-Acetyl-Galactosaminidase	-	L-Malate assimilation	-
Beta-N-Acetyl-Glucosaminidase	-	L-Proline Arylamidase	-
Beta-Xylosidase	-	L-Pyrrolydonyl Arylamidase	-
Citrate	+	Lipase	-
Coumarate	+	Lysine Decarboxylase	-
D-Cellibiose	-	Malonate	+
D-Glucose	+	O/129 resistance	+
D-Maltose	+	Ornithine Decarboxylase	+
D-Mannitol	+	Palatinose	-
D-Mannose	+	Phosphatase	+
D-Sorbitol	+	Saccharose/Sucrose	-
D-Tagatose	-	Succinate alkalinisation	+
D-Trehalose	+	Tyrosine Arylamidase	+
Coumarate	+	Urease	-
D-Cellibiose	-	5-Keto-D-Gluconate	-
D-Glucose	+		

+: positive result, -: negative result.

Table II.ii. Results of susceptibility to antimicrobial agents of isolates of *Salmonella enterica* from cases of diarrhoea in lambs.

Antimicrobial agent	Susceptibility result
Ampicillin	I
Gentamicin	S
Enrofloxacin	S
Erythromycin	R
Cefquinome	I
Trimethoprime + Sulfadiazine	R

R: resistant, I: intermediate, S: susceptible

Finally, results of both serotyping procedures revealed that both isolates were *Salmonella enterica* subsp. *diarizonae* 61:k:1,5,(7).

Follow-up actions and further monitoring of the farm

Materials and methods

Based on the results of susceptibility to antimicrobial agents, enrofloxacin was prescribed for administration to lambs with clinical signs at a dose rate of 5 mg kg^{-1} once daily for a 5-day course. Metaphylactic treatment was also prescribed to clinically healthy newborn lambs (2nd day of life), at the above dose rate, for a 2-day course.

Thereafter, the situation was monitored by collecting sequential faecal samples from lambs that had clinical signs and had been administed antibiotic treatment (n = 6), as well as from clinically healthy lambs (n = 6). Samples were also collected from dams of lambs with clinical signs (n = 6). Starting 15 days after the end of administration of the antimicrobial agent, samples were collected at weekly intervals for up to 42 days.

Samples were examined microbiologically for presence of *Salmonella* spp. A 1:20 faecal suspension was prepared into buffered peptone water and incubated for 18 h at 37 ^{o}C. Subsequently, 100 μL of the incubated peptone water were plated onto modified semi-solid Rappaport Vassiliadis agar, for aerobic incubation at 41.5 ^{o}C for up to 48 h. If smooth white colonies were observed on the media, a small quantity was collected by means of a microbiological loop and plated onto Xylose Lysine Deoxycholate agar plates, for 24 h incubation at 37 ^{o}C. Black clolonies developed in XLD agar plates were considered to be *Salmonella* spp.

Isolates identified as *Salmonella* by using the above procedure, were then examined by means of API rapid identification system.

Results

S. enterica was isolated from one sample collected from lambs that had had clinical signs, from no samples collected from clinically health lambs and from one sample from the dam of a lamb that had had clinical signs. Both samples that yielded the organism, had been collected on the 2nd follow-up sampling, i.e., 22 days after the end of administration of the antimicrobial agent.

C. BACTERIOLOGICAL EXAMINATION OF FAECAL SAMPLES FROM LAMBS WITH DIARRHOEA IN 21 SHEEP FARMS IN CENTRAL GREECE

Materials and methods

Sheep farms and animals

The work was carried out from late autumn 2014 to early spring 2015, during the lambing and lactation period in 21 sheep farms in central Greece. Sheep management in these farms were of the semi-intensive or semi-extensive type. A variety of indigenous (e.g., Karagouniko, Chios) or imported (Lacaune, Assaf) breed animals were present in the flocks. As per principal production system in Greece, lambs sucked their dams until 45- to 55-day-old, at which age they would go for slaughter.

Criteria for inclusion of flocks in the study were: (i) flock size over 150 animals and (ii) presence of clinical signs of diarrhoea in lambs in the flock. Farms were visited for sample collection. Faecal sample collection from lambs was performed within 48 h of onset of clinical signs and prior to administration of any antimicrobial or antiparasitic medication to lambs.

Samplings

In total, faecal samples were collected from 119 newborn lambs younger than two weeks. Within each flock, samples were collected from three to six lambs. Initially, a swab was inserted (1.0-1.5 cm) into the rectum of each animal, swirled and then removed. Further, 20 g of faeces were collected into the gloved hand of the investigator. Swabs were placed into transport medium and faeces were maintained in cold storage until transport to the laboratory, which took place within 4 hours maximum.

Laboratory examinations

In all cases, rectal swabs and faecal samples were processed within 24 h of collection. Samples were processed for presence of microbial or parasitic agents by using the techniques described below.

At first, rectal swabs were cultured 5% sheep blood agar and McConkey agar and incubated at 37 °C for up to 48 h. All morphologically different colonies on each plate were cultured on tryptic soya agar to recover pure colonies. Conventional microbiological techniques, the API rapid identification system and the Vitek 2 system were used for identification of organisms. Swabs were also cultured on CDC Anaerobe 5% Blood Sheep agar (BD) for anaerobic incubation up to 72 h for possible isolation of *Clostridium perfringens*. For isolation of *Salmonella* spp., the procedure described in ISO: 6579:2002 protocol was followed, starting with a 20 g of faeces and using buffered peptone water as recovery medium, a Modified Semisolid Rappaport-Vassilliadis Medium (MSRV) and a Xylose-Lysine Deoxycholate agar (XLD) agar.

For detection of *Group A Rotaviruses*, *Coronavirus* and *Adenovirus* in faecal samples, commercially available rapid test kits (VIKIA® Rota-Adeno and Rota-Corona-Parvo Quicking®), which detected viral antigens, were used, as per manufacturers' specifications. When *Rotavirus* was detected in a samples, the presence was confirmed by using a RT-PCR protocol as previously described (World Health Organization 2009); faecal homogenates were prepared in phosphate buffer saline and RNA extraction was performed using Ambion RNA kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' protocol. The extracted double stranded RNA was denaturated at 97 °C for 5 min and placed immediately on ice; a two-step multiplex RT-PCR was performed to characterise G and P types of the *Rotavirus* strains, based on previously published terms and conditions (World Health Organisation 2009); amplicons corresponding to each G and P type were visualised under ultra-violet light on 2% agarose gel. Finally, for detection of *Cryptosporidium* spp. in faecal samples, a smear from each sample was stained by means of the modified Ziehl-Neelsen technique; additionally, the flotation method using ZnSO4 33.2% solution was performed to detect any *Giardia* spp. oocysts (Ministry of Agriculture, Fisheries and Food 1984).

Results

Clinical findings

Clinical severity of diarrhoea in lambs was of mild intensity to profuse, intense disease with dehydration of the animal. Median morbidity rate in flocks into the study was 15% (range: 10% - 60%); median fatality rate was 12% (range: 0% - 40%).

Pathogen detection in samples from lambs

In total, 229 bacterial isolates were recovered from faecal samples of lambs. *Salmonella enterica* subsp. *diarizonae* or any other *Salmonella* sp. was not isolated from any sample from any farm. Prevalence of infection was 0.000 (95% confidence intervals: 0.000 - 0.031). *Escherichia coli*, in pure or mixed culture, was the most frequently isolated bacterial pathogen: 101 isolates were recovered from 67 lambs (one isolate from 39 lambs, two isolates from 22 lambs and three isolates from 6 lambs). Other bacteria isolated were *Enterobacter* spp., *Proteus* spp., *Klebsiella* spp., *Cl. perfringens*, *Citrobacter freundii* (Table II.iii). Group A *Rotavirus* was detected in samples from three lambs (2.5%) in two different flocks (9%); the G10P[8] combination was determined in one sample, i.e. regarding one *Rotavirus* strain, while the other two *Rotavirus* strains (both from the same flock) remained untyped. *Coronavirus* or *Adenovirus* were not detected in any sample from any flock. Finally, *Cryptosporidium* spp. oocysts were observed in smears of faecal samples from 13 lambs (10.5%), collected from 11 flocks (50%), whilst no *Giardia* oocysts have been detected.

Table II.iii. Frequency of isolation of bacteria from faecal samples from lambs with diarrhoea.

	Frequency of isolation [n (proportion)]		
Microorganism	**Isolates**	**Lambs**	**Farms**
Escherichia coli	101 (44.1%)	67 (56.3%)	21 (100.0%)
Enterobacter aerogenes	39 (17.0%)	39 (32.8%)	8 (38.1%)
Proteus vulgaris	23 (10.0%)	23 (19.3%)	9 (42.9%)
Klebsiella pneumoniae	20 (8.7%)	20 (16.8%)	8 (38.1%)
Proteus mirabilis	16 (7.0%)	16 (13.4%)	7 (33.3%)
Clostridium perfringens	14 (6.1%)	14 (11.7%)	3 (14.3%)
Citrobacter freundii	9 (4.0%)	9 (7.5%)	3 (14.3%)
Klebsiella oxytoca	4 (1.7%)	4 (3.3%)	1 (4.7%)
Enterobacter cloacae	3 (1.3%)	3 (2.5%)	1 (4.7%)
Total	229	119	21

CHAPTER III

EXPERIMENTAL WORK IN THE POTENTIAL ROLE OF *SALMONELLA ENTERICA* SUBSP. *DIARIZONAE* FOR THE DIARRHOEIC SYNDROME OF LAMBS

A. INTRODUCTION

Salmonella enterica subsp. *diarizonae* is regularly isolated from faecal samples and gastrointestinal tract tissue samples from apparently healthy lambs and adult sheep. The organism has been also detected in such samples from lambs suffering with diarrhoea, potentially leading in a diagnostic problem regarding the causal agent(s) of the problem. Despite relevant studies in the recent past, the possible role of the pathogen in the aetiology of diarrhoeic syndrome in lambs remains unclear.

Objective of this experimental work was the evaluation of the potential role of *S. enterica* subsp. *diarizonae* in the diarrhoeic syndrome in lambs and the investigation of facets of the pathogenesis of the infection.

B. MATERIALS AND METHODS

Animals

In total, 16 lambs from 8 ewes were employed and enrolled in the study on the first day of life. Of these, 12 lambs were challenged with *Salmonella enterica* subsp. *diarizonae*. The dams of the lambs had been housed throughout their gestation and were provided with a commercial concentrate feed plus hay and barlay straw. Throughout the study, ewes were housed; during the final month of gestation and after lambing, ewes were housed individually; in the latter case, lambs were also penned with their respective dam.

Two examinations of blood samples for concentrations of β-hydroxybutyrate (Barbagianni et al. 2015) did not reveal any problems: in all animals, concentrations were always below 0.95 mmol L^{-1}; further, examination of serum blood samples by using ELISA tests with commercially available kits, for presence of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* (ID Screen® Paratuberculosis Indirect; ID VET, Grabels, France), *Small Ruminant Lentivirus* (ID Screen® MVV / CAEV Indirect; ID VET) and *Bluetongue Virus* (ID Screen® Bluetongue Competition, ID VET) (Köhler et al. 2008, Vandenbussche et al. 2008, Michiels et al. 2018), also did not reveal any problems: in no ewe, antibodies of above pathogens were detected by any of these tests.

Lambs were included in the study after a detailed clinical examination was performed within 6 hours after birth, if (a) no abnormal findings were clinically evident and (b) results of bacteriological examination of faecal samples, performed as detailed below for detection of *Salmonella* spp., were not positive.

The experiment was performed under a licence issued by the Veterinary Authority of the Region of Thessaly, which was the competent authority for this matter.

Inoculation procedure

The lambs were challenged with a *Salmonella enterica* subsp. *diarizonae* that had been isolated during the investigation of the outbreak of diarrhoeic syndrome in a sheep farm (Chapter II). Inoculation of lambs was performed on the 1st day of life (D0).

For inoculation, the challenge isolate was cultured in brain heart broth for 12 h at 37 °C. The culture was centrifuged and the sediment diluted into 20 mL phosphate-buffer saline pH 7.3 (PBS).

A quantity of 10 mL of PBS was aspirated with a sterile syringe and, through use of a sterile plastic gastric catheter, was slowly introduced into the abomasum of the newborn lambs, by following the standard principles of administration of oral solutions to lambs (Henderson 2007). Lambs were maintained at that position for 3 min after end of the procedure. The inoculum varied from 0.75×10^9 to 1.80×10^9 c.f.u., as estimated by the method of Miles and Misra (1938).

Four lambs received 10 mL of sterile PBS by using the above technique and were used as uninfected controls.

Examination of animals and samplings

Lambs

On D0, but before challenge, a detailed clinical examination was carried out in lambs. Blood samples were collected for haematological examination. Faecal and buccal swab samples were collected for bacteriological examination for detection of *Salmonella* spp.

Post-challenge, clinical examinations and sample collection as above were performed 6 hours (D0 + 6 h), 12 hours (D0 + 12 h), 1 day (D1) and 2 (D2), 4 (D4), 7 (D7), 10 (D10), 14 (D14) and 21 (D21) days. On D1 and thereafter, lambs were euthanised (n = 1 on D1 and D2, n = 2 on subsequent time-points). The uninfected controls were euthanised on D4, D10 and D21.

A detailed post-mortem examination was performed in all euthanised lambs. Tissue samples from the abomasum, the small intestine, the liver and the gallbladder were collected for bacteriological examination and for detection of *Salmonella* spp. genetic material by molecular techniques. Further, tissue samples from the abomasum, the small intestine and the mesenteric lymph nodes were collected for histopathological examination.

Ewes

On the same occasions as above, faecal swab and milk samples were collected from the ewes for bacteriological examination. Milk samples were collected aseptically, separately from each of the two mammary glands of each ewe (Fthenakis 1994).

Laboratory examinations

Haematological examination

Samples for haematological examination were mixed by gentle repeated inversions for several seconds to avoid coagulation. They were processed within 30 min after collection. Initially, blood smears were prepared and kept dry at room temperature. A complete blood count was performed by a previously evaluated in ovine haematology automated haematological analyser (Abbott Cell-Dyn 3500 System; Abbott, Abbott Park, IL, USA) (Athanasiou et al. 2013). The following parametres were determined: haematocrit, erythrocyte count, haemoglobin concentration, mean corpuscular volume, mean corpuscular haemoglobin concentration, total leucocyte count and thrombocyte count. Blood smears were evaluated for leucocyte type differentiation and detection of potential presence of morphological abnormalities.

Bacteriological examination

Faecal swab samples from lambs and ewes were processed as detailed previously for isolation of *Salmonella* (Chapter II); for recovery of the organism, each swab was initially immersed into 1 mL of buffered peptone water. Buccal swabs from lambs were also processed similarly. For milk samples from ewes, a volume of 1 mL of milk was mixed with 10 mL of buffered peptone water, which was followed by the same procedure as above.

Tissue samples from lambs collected during post-mortem examination were washed with PBS and, then, were homogenised (10 g of tissue sample with 50 mL of sterile PBS blended for 3 min) in a tissue blender (Mixwel; Alliance Bio Expertise, Guipry, France); then, of the resulting fluid, 20 mL were added into 200 mL of buffered peptone water, which was followed by the same procedure as above.

Molecular examination for presence of *Salmonella* spp.

DNA extraction

Faecal swab samples were washed with 0.2 mL of PBS into a sterile 2 mL Eppendorf tube. Then, 0.02 mL of proteinase K (PureLink® Genomic DNA Kit K 1820-01 [Life Technologies]) and 0.02 mL of RNAse A (PureLink® Genomic DNA Kit K 1820-01 [Life Technologies] were added therein. After thorough mixing in a Vortex equipment for 1 min and incubation for 2 min at room

temperature, 0.2 mL of genomic lysis buffer (PureLink® Genomic DNA Kit K 1820-01 [Life Technologies, Karlsbad, USA]) was added in the mixture. The new mixture was incubated at 55 °C for 10 min, which was followed by addition of 0.2 mL of pure ethanol (Sigma-Aldrich, Saint Louis, USA) and thorough mixing. The lysate was transferred to a spin cartridge collection tube, which was centrifuged for 30 s at 12,000 *g* at room temperature.

Buccal swab samples were washed with 0.4 mL of PBS into a sterile 2 mL Eppendorf tube. Then, 0.02 mL of proteinase K was added therein. After thorough mixing in a Vortex equipment for 1 min, a volume of 0.4 mL was transferred to a new sterile 2 mL Eppendorf tube, into which 0.4 mL of genomic lysis buffer was added. The new mixture was incubated at 55 °C for 10 min and then centrifuged for 1 min at 8,000 *g* at room temperature. Finally, addition of 0.4 mL of pure ethanol and thorough mixing were performed. The lysate was transferred to a spin cartridge collection tube, which was centrifuged for 30 s at 12,000 *g* at room temperature.

A volume of 0.2 mL of a milk whey sample (obtained after centrifugation of the original milk samples and aspiration of the relevant fraction of the product) was mixed with 0.02 mL of proteinase K and 0.02 mL of RNAse A into a sterile 2 mL Eppendorf tube. After thorough mixing in a Vortex equipment for 1 min and incubation for 2 min at room temperature, 0.2 mL of genomic lysis buffer was added in the mixture. The new mixture was incubated at 55 °C for 10 min, which was followed by addition of 0.2 mL of pure ethanol and thorough mixing. The lysate was transferred to a spin cartridge collection tube, which was centrifuged for 30 s at 12,000 *g* at room temperature.

Tissue samples from lambs were prepared for molecular examination as follows. Initially, 0.05 g of tissue sample was placed into an Eppendorf tube with 0.5 mL of PBS and subjected to mixing in a Vortex equipment (Velp Scientifica, Usmate, Italy) for 3 min. Then, 0.5 mL of the mixture was transferred to a new Eppendorf tube for DNA extraction. Into this, 0.180 mL of digestion buffer (PureLink® Genomic DNA Kit K 1820-01 [Life Technologies, Karlsbad, USA]) and 0.02 mL of proteinase K were added and the mixture was incubated at 55 °C for 2 h; occasionally (once or twice every hour); the final product was subjected to mixing in a Vortex equipment for 1 min. At the end of the incubation period, 0.02 mL RNase A was added and the mixture was left at room temperature for 2 min. The resulting lysate was centrifuged for 5 min at 12,000 *g* at room temperature. The supernatant was transferred to a new microcentrifuge tube and 0.01 mL 10% SDS was added, followed by mixing in a Vortex equipment for 5 s. Then, a volume of 0.2 μL binding buffer (PureLink® Genomic DNA Kit K 1820-01 [Life Technologies]) was added to the lysate, which was again followed by mixing in a Vortex equipment for 5 s. The lysate was incubated at 70 °C for 10 min, after which a volume of 0.2 mL of pure ethanol was added, again followed by mixing in a Vortex equipment for 5

s. The lysate was transferred to a spin cartridge collection tube, which was centrifuged for 30 s at 12,000 *g* at room temperature.

Thereafter, in all above cases, the collection tube was discarded and a new sterile tube was placed to spin catridge tube. A volume of 0.5 mL wash buffer I (PureLink® Genomic DNA Kit K 1820-01 [Life Technologies]) was added and centrifuged for 30 s at 12,000 *g* at room temperature. The fluid collected to the wash tube was discerded and the procedure was repeated once. A volume of 0.5 mL wash buffer II (PureLink® Genomic DNA Kit K 1820-01 [Life Technologies]) was added and centrifuged for 30 s at 12,000 *g* at room temperature. Again, the fluid collected to the wash tube was discarded and the procedure was repeated once. Finally, the tube was centrifuged for 2 min. at 12,000 *g* at room temperature. The binded DNA was eluted using 0.2 mL elution buffer (PureLink® Genomic DNA Kit K 1820-01 [Life Technologies]) and the mixture remained at room temperature for 1 min, subsequently then it was centrifuged for 90 s at 12,000 *g* at room temperature. The DNA was splitted in aliquots of 0.005 mL and stored at -20 ^{o}C.

PCR amplification

Presence of *Salmonella* spp. *invA* gene was detected by simple PCR assay. Details of primers and conditions employed are in Table III.i. Amplification was performed in a PT-100 Thermocycler (MJ Research Inc., St Bruno, Canada). Reactions were performed in a total volume of 0.05 mL PCR mixture, containing 0.045 mL of Platinum PCR SuperMix (Applied Biosystems, Foster City, USA) and approximately 150 ng of the extracted DNA. The thermal cycling procedure consisted of a pre-denaturation step at 95 ^{o}C for 2 min, 35 cycles of denaturation at 94 ^{o}C for 1 min, annealing for 1 min and extending at 72 ^{o}C for 45 s and a final elongation step at 72 ^{o}C for 7 min. Subsequently, 0.005 mL of each product was analysed by electrophoresis on 1.5% agarose gel stained with ethidium bromide (100 mL 1 × TBE buffer [DGel Electrosystem, Montreal, Canada], 2 g agarose [NIPPON Genetics, Tokyo, Japan], 0.005 mL ethidium bromide [Sigma-Aldrich]) and observed at ultraviolet light. Each product equal in size to the expected amplicon was considered as positive.

Table III.i. Primers used and work conditions undertaken for detection of *Salmonella* spp. *invA* gene in DNA extract from tissue samples from lambs.

Primer sequence	Concentration (μM)	Product size (bp)	AT[a] (^{o}C)	Reference
Fw-GTGAAATTATCGCCACGTTCGGGCAA Rv-TCATCGCACCGTCAAAGGAACC	0.25	284	55.0	Rahn et al. 1992

[a] AT: annealing temperature.

Histopathological examination

Tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Haematoxylin and eosin (HE) standard staining procedures were performed for histopathological studies.

Data management and analysis

All data were entered into Microsoft Excel and analyzed using IBM SPSS Statistics (ver. 21) (IBM; Armonk, NY, USA).

For estimation of incidence rates, we took into account that a lamb might change from being infected to not being infected and vice-versa; during the interval between sampling points, it was not possible to know what had happened between the two sampling points, i.e. how many cases of infection and 'cures' there might have occurred. Therefore, the following definitions were made (Mavrogianni et al. 2007).

• "Isolation of bacteria" was equivalent to "infection with"; "isolation of bacteria from a sample" was equivalent to "infection of the tissue from which the sample had been collected".

• On a particular sampling point, an animal was defined as being 'at risk of developing infection', if it had no infection on the previous sampling point.

• On the subsequent sampling point, this animal could be either 'healthy' (in which case it was still at risk to becoming infected) or 'with infection' (in which case it was not at risk).

• On subsequent sampling points, if this animal was 'healthy', then it was again 'at risk'.

• If an animal was detected to be healthy on one sampling point but with infection on the next one, then infection was considered to have taken place half-way between the two sampling points; if an animal was detected with infection on one sampling point but healthy on the next one, then infection was deemed to have been eliminated half-way between the two sampling points.

• If an animal was detected with infection on two consecutive sampling points, then it was considered to have been with infection throughout the time between those two time points; conversely, if an animal was found healthy on two consecutive sampling points, then it was considered to have been healthy throughout the time between those two time points.

Based on the above, it was possible to calculate incidence rates of the various infections. Further, it was possible to estimate the length of time for which an animal was at risk before it became infected, as well as the length of time that an animal had been infected. Incidence rate was defined

as the proportion of animals at risk, which developed the condition when the time at risk was the same in each group.

Linear mixed models were used in analysis to account for repeated measures of values of haematological parametres over the course of the study. Time points of collecting data were selected as within-subjects variables and group allocation as between-subject factor. Independent variables (fixed effects) included study group, sampling point and a sampling point by study group interaction.

The various associations were evaluated in a table of cross-categorised frequency data by use of the Pearson chi-square test or the Fisher exact test as appropriate.

C. RESULTS

Clinical findings

Lambs

All lambs were clinically healthy before challenge. Only one of the inoculated lambs developed clinical signs within 12 h after challenge (incidence rate: 0.071, 95% confidence interval: 0.013 – 0.315); the clinical signs lasted until D2 and included increased rectal temperature (> 42.0 ^{o}C), diarrhoea (watery and yellow-coloured), dullness and depression, recumbency, increased respiratory rate (> 55 min^{-1}). The control lambs remained healthy throughout the study (P = 0.76 for presence of clinical findings between inoculated and control lambs).

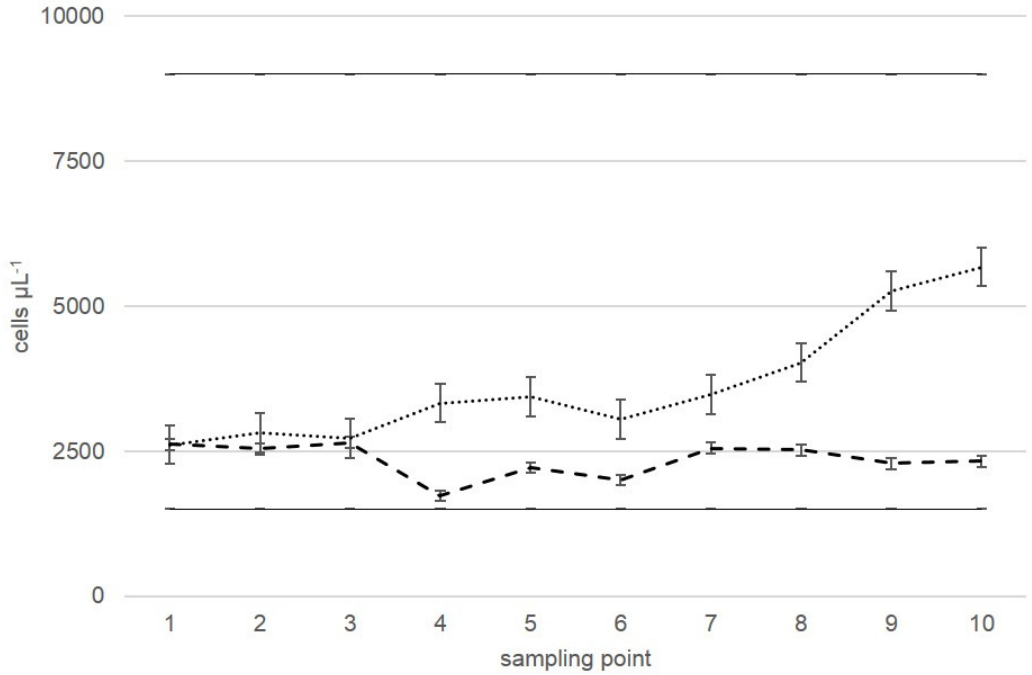
Ewes

No ewe showed any clinically evident abnormalities during the study.

Haematological findings

A significant difference was seen only in lymphocyte numbers after D4, which were higher in inoculated lambs (P < 0.042). Nevertheless, even in these animals, the findings were within the proposed respective reference range (reference range taken into account as discussed by Roger [2008]). No morphological abnormalities were detected in leucocytes. Details are in Figure III.1 and Table III.ii.

Figure III.1. Median number of lymphocytes in blood samples from lambs inoculated with *S. enterica* subsp. *diarizonae* (dotted line) or control lambs (dashed line) for 21 days after birth.



Straight lines: reference values (Roger 2008).
Sampling points: 1: before challenge, 2: 6 h, 3: 12 h, 4: 1 d, 5: 2 d, 6: 4 d, 7: 7 d, 8: 10 d, 9: 14 d, 10: 21 d after challenge.

Table III.ii. Haematological findings (median values) in lambs orally inoculated with *S. enterica* subsp. *diarizonae*.

(a) control lambs

	Sampling point									
Parametre	**D0**	**D0 + 6 h**	**D0 + 12 h**	**D1**	**D2**	**D4**	**D7**	**D10**	**D14**	**D21**
Haematocrit (%)	26.1	28.0	28.1	28.3	27.7	28.9	29.1	29.2	28.3	28.7
Erythrocytes ($\times 10^6$ cells μL^{-1})	10.2	10.4	11.1	11.4	10.7	11.0	11.9	9.8	9.9	10.1
Haemoglobin (g dL^{-1})	11.1	10.7	10.9	11.1	10.6	11.4	12.0	11.4	10.5	10.5
MCV (fL)	45.2	44.6	44.2	45	44.2	41.2	41.7	40.4	40.7	41.3
MCHC (g dL^{-1})	12.4	12.7	12.9	12.4	11.0	11.0	11.6	10.5	9.6	10.3
Total leucocytes (cells μL^{-1})	6425	7010	8200	5350	4370	5000	5085	4650	4105	9490
Neutrophils (cells μL^{-1})	3300	4040	5280	3400	1800	730	2255	1845	1580	3250
Neutrophils (% leucocytes)	51.0	57.6	64.4	63.5	40.6	14.6	44.4	40.0	38.2	34.4
Lymphocytes (cells μL^{-1})	2620	2540	2650	1730	2215	2000	2555	2520	2290	2325
Lymphocytes (% leucocytes)	40.9	36.2	32.3	32.3	51.2	80.0	50.0	53.6	56.2	55.8
Monocytes (cells μL^{-1})	65	20	20	20	180	20	100	100	50	105
Monocytes (% leucocytes)	0.9	0.3	0.2	0.4	4.0	0.4	2.0	2.2	1.2	1.1
Eosinophils (cells μL^{-1})	285	260	110	70	45	40	45	85	120	600
Eosinophils (% leucocytes)	4.6	3.7	1.3	1.2	1.0	0.8	0.9	2.1	2.2	6.4

Table III.ii. (continued).

(a) control lambs (continued)

Parametre	Sampling point									
	D0	D0 + 6 h	D0 + 12 h	D1	D2	D4	D7	D10	D14	D21
Basophils (cells μL^{-1})	110	100	90	80	70	180	95	55	45	100
Basophils (% leucocytes)	1.7	1.4	1.0	1.5	1.7	3.6	1.8	1.2	1.1	1.0
Thrombocytes (cells μL^{-1})	763	893	844	922	1030	1045	1770	1503	1167	1070

(b) infected lambs

Parametre	Sampling point									
	D0	D0 + 6 h	D0 + 12 h	D1	D2	D4	D7	D10	D14	D21
Haematocrit (%)	33.8	30.6	28.9	30.0	30.4	28.1	28.9	28.6	27.8	34.4
Erythrocytes ($\times 10^6$ cells μL^{-1})	8.22	7.59	7.32	7.68	7.66	7.21	7.73	8.07	8.7	10.5
Haemoglobin (g dL^{-1})	10.53	9.81	9.45	9.37	9.69	8.97	9.1	8.84	8.3	10.3
MCV (fL)	40.35	39,66	38.98	39.17	39.55	38.70	35.13	35.2	32.7	33.2
MCHC (g dL^{-1})	12.8	12.7	12.7	12.43	12.7	12.3	11.9	10.9	9.8	10.0
Total leucocytes (cells μL^{-1})	4740	4650	4720	5270	5300	4970	4935	5395	7280	10725
Neutrophils (cells μL^{-1})	2100	1945	1460	1550	1790	1480	1230	990	2330	3475
Neutrophils (% leucocytes)	42.1	37.9	29.1	31.8	32.2	29.5	27.2	17.9	27.5	34.3

Table III.ii. (continued).

(b) infected lambs (continued)

Parametre	Sampling point									
	D0	D0 + 6 h	D0 + 12 h	D1	D2	D4	D7	D10	D14	D21
Lymphocytes (cells μL^{-1})	2610	2815	2720	3330	3440	3050	3475	4025	5260	5675
Lymphocytes (% leucocytes)	49.7	55.3	63.2	63.1	60.4	60.8	66.1	74.8	63.8	53.0
Monocytes (cells μL^{-1})	155	120	60	60	245	80	70	115	190	150
Monocytes (% leucocytes)	2.6	3.2	1.6	1.5	4.0	1.6	1.3	1.8	2.1	1.4
Eosinophils (cells μL^{-1})	50	70	40	60	40	50	50	35	130	1140
Eosinophils (% leucocytes)	1.0	1.3	1.0	1.5	0.7	1.0	0.9	0.8	1.7	9.1
Basophils (cells μL^{-1})	90	120	100	90	90	110	120	95	90	185
Basophils (% leucocytes)	1.9	2.1	2.0	2.0	1.9	2.2	2.6	1.7	1.2	1.2
Thrombocytes (cells μL^{-1})	545	556	512	549	598	934	1028	1146	1151	1082

D0: day of inoculation; D1, D2, D3, D4: days after inoculation.
MCV: mean corpuscular volume, MCHC: mean corpuscular haemoglobin concentration.

Bacteriological findings

Faecal swab samples from lambs

Salmonella was not isolated from any faecal sample from any lamb before oral inoculation. After inoculation, the challenge organism was isolated from faecal samples of all lambs (1.000) at least once; intermittent bacterial isolation was recorded in 3 lambs. In total, the organism was isolated from 45 of 77 (0.584) samples collected from the lambs post-inoculation. Median time of first bacterial isolation was 6 h and median duration of bacterial isolation was 2.4 days post-inoculation.

The organism was not isolated from any faecal sample from the uninfected control lambs.

Buccal swab samples from lambs

Salmonella was not isolated from any buccal sample from any lamb before oral inoculation. After inoculation, the challenge organism was isolated from buccal samples from 7 lambs (0.583) at least once. In total, the organism was isolated from 10 of 77 (0.130) samples collected from the lambs post-inoculation. Median time of first bacterial isolation was 1 d and median duration of bacterial isolation was 0.8 day post-inoculation.

The organism was not isolated from any buccal sample from the uninfected control lambs.

Faecal swab samples from ewes

Salmonella was not isolated from any faecal sample from any dam of inoculated lambs, before or after inoculation of these lambs. Further, the organism was not isolated from any sample from the dams of the uninfected control lambs.

Milk samples from ewes

Salmonella was not isolated from any milk sample from any dam of inoculated lambs, before their inoculation of these lambs. Thereafter, *Salmonella* was isolated from two milk samples, collected from the same ewe, dam of an inoculated lamb, on two consecutive sampling occasions (D4, D7). From buccal samples of the lamb of that ewe, *Salmonella* was also consistently isolated from D2 to D7. Subsequent detailed identification of the organism confirmed its identity as *S. enterica* subsp. *diarizonae*.

The organism was not isolated from any milk sample from the dams of the uninfected control lambs.

Tissue samples from lambs

Salmonella was isolated from tissue samples of 9 lambs. In total, the organism was isolated from 15 of 48 (0.313) tissue samples collected from the euthanised lambs post-inoculation. The organism was isolated from the small intestine of 6 lambs (0.500), the abomasum of 4 lambs (0.333), the liver of 3 lambs (0.250) and the gallbladder of 2 lambs (0.167). There was no association between concurrent isolation of *Salmonella* from faecal samples and from small intestine or any tissue sample from the same lamb on the day of euthanasia ($P > 0.22$) (Table III.iii).

The organism was not isolated from any tissue sample from the uninfected control lambs.

Table III.iii. Association between concurrent isolation of *Salmonella* on the day of euthanasia from faecal samples and from tissue samples from the same lamb.

		Isolation in faecal samples	
		+	**-**
Isolation in small intestine tissue samples	**+**	2	4
	-	0	6
Isolation in any tissue samples	**+**	3	8
	-	1	0

Molecular findings

Detailed results of detection of the *invA* gene of *Salmonella* spp. in samples from inoculated lambs or their dams are in Table III.iv. In total, the *invA* gene was detected in samples from all inoculated lambs and from samples of two of their dams. In all samples, of all tissues, that had yielded *Salmonella* at the microbiological examination, the *invA* gene was also detected (1.000).

Median duration of detection of the gene was 5.5 days (0.38 – 17.5) in faecal samples, 1.3 days (0 – 4.8) in buccal samples and 0 days (0 – 5.5) in milk samples.

The gene was not detected in any sample from the uninfected control lambs.

Table III.iv. Detection of the *invA* gene from various samples collected from lambs inoculated with *S. enterica* subsp. *diarizonae* and their dams.

Animal	Samples	No. of animals in which detected	No. of samples in which detected
Lambs	Faeces	12 / 12 (1.000)	70 / 77 (0.909)
Lambs	Buccal cavity	9 / 12 (0.750)	24 / 77 (0.312)
Ewes	Milk	2 / 6 (0.333)	3 / 78 (0.038)[1]
Lambs	Small intestine	12 / 12 (1.000)	12 / 12 (1.000)
Lambs	Abomasum	8 / 12 (0.667)	8 / 12 (0.667)
Lambs	Liver	12 / 12 (1.000)	12 / 12 (1.000)
Lambs	Gallbladder	8 / 12 (0.667)	8 / 12 (0.667)

1. Corresponding to 3 / 39 sampling occasions (0.078).

Pathological findings

Gross pathological findings

During post-mortem examination, there was swelling of the abomasal and the intestinal wall; the latter was also turgid. In some cases, there was fibrinohaemorrhagic enteritis. Lesions were located more prominently at the ileum, especially in lambs euthanised up to D7; in lambs euthanised afterwards, there were lesions also in the jejunum and colon; in all cases, no macroscopic lesions were seen in the duodenum. The mesenteric lymph nodes were enlarged.

Histopathological findings

There was abomasitis with subepithelial presence of eosinophils, lymphocytes and plasma cells. This was a consistent finding observed in all inoculated lambs, although there was a varying degree of the inflammation (mild to moderate) between animals.

In the small intestine, there was a variety of lesions when compared to healthy tissue (Figure III.2). At the early stage post-inoculation (up to D10), salient lesions included distension and oedema of intestinal villi (Figure III.3), leucocytic infiltration (macrophages, neutrophils, lymphocytes, plasma cells) (Figures III.4 and III.5) and hyperplasia of lymphoid nodules with apparent germinal centres (Figure III.6). At later stages (D10 and thereafter), the lymphoid tissue of the intestine (Peyer's patches) was consistently observed to be atrophied and / or degenerated (Figure III.7); there was also marked subepithelial infiltration of lymphocytes, plasma cells and eosinophils.

Figure III.2. Small intestine: normal lymphofollicular tissue (HE, bar 500 μm).

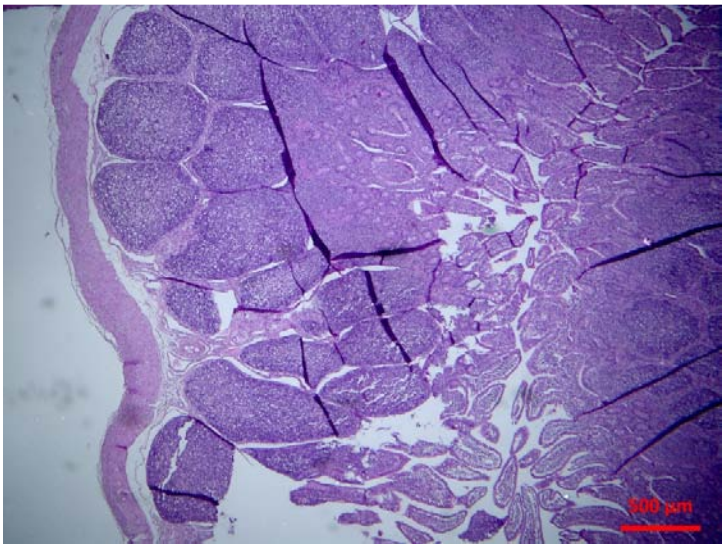


Figure III.3. Small intestine mucosa: dilatation of lymphatic vessels of small intestine (arrows) (HE, bar 100 μm).

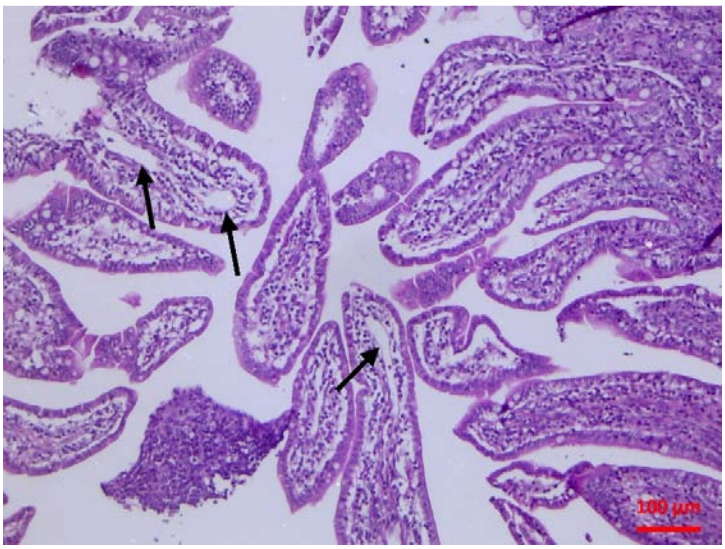


Figure III.4. Small intestine mucosa: neutrophilic infiltration (black arrows) (HE, bar 20 μm).

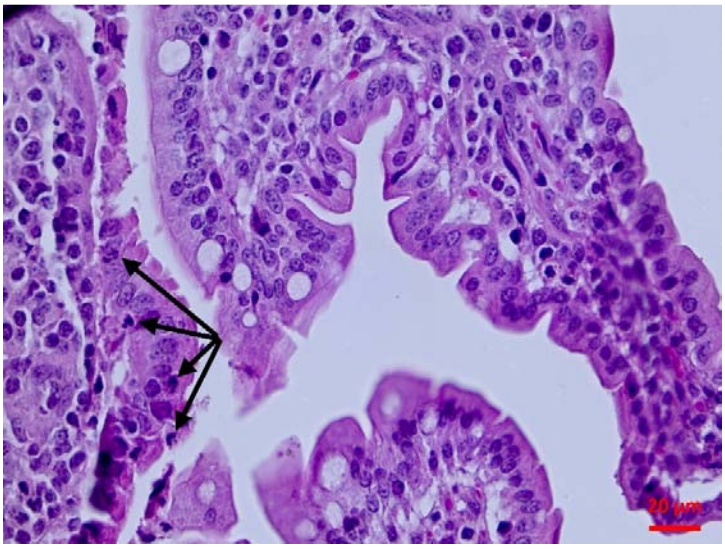


Figure III.5. Small intestine mucosa: lymphocytic infiltration of the villous lamina propria (arrow) (HE, bar 100 μm).

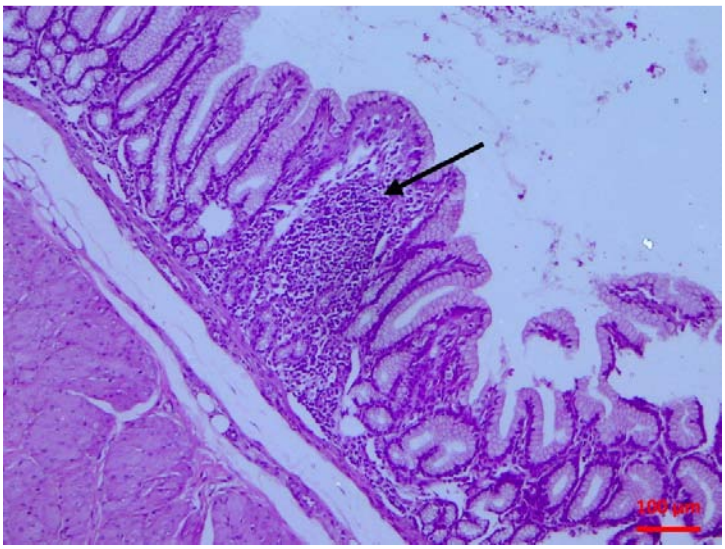
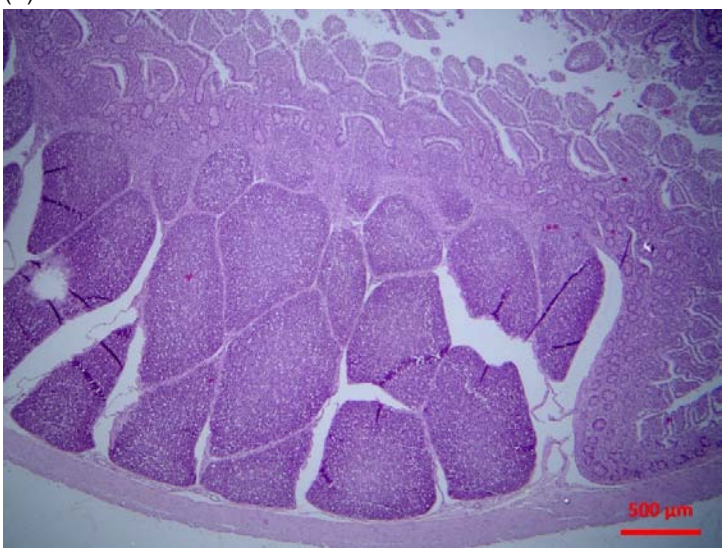


Figure III.6. Small intestine: mild (a) to moderate (b) lymphofollicular hyperplasia (HE, bar 500 μm).

(a)



(b)

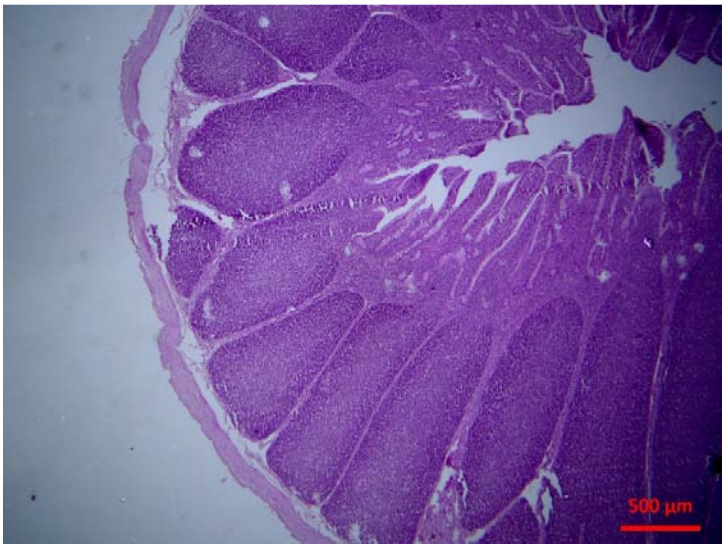
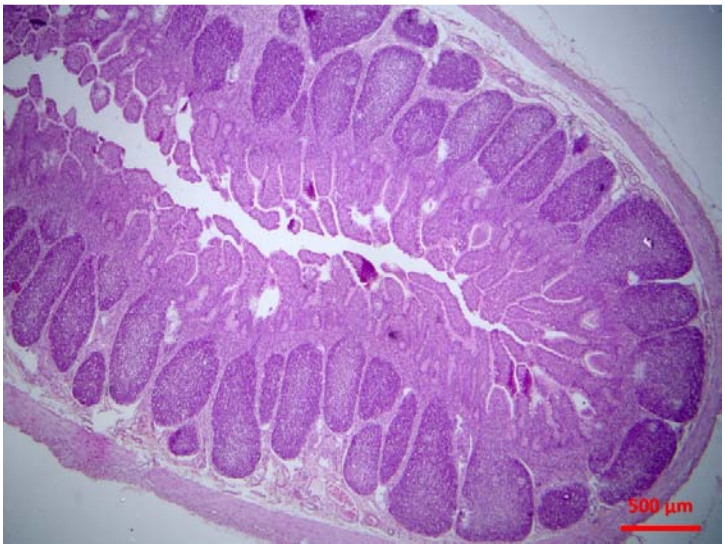


Figure III.7. Small intestine: mild lymphofolicullar atrophy (HE, bar 500 μm).



GENERAL DISCUSSION

Introduction

Salmonella enterica subsp*. diarizonae* is the most commonly detected *Salmonella* serovar in sheep. The organism is considered to be host-adapted. In most cases, infections caused by this organism are endemic in flocks and remain subclinical (Soren et al. 2015). Moreover, the organism has been sporadically associated with a variety of clinical conditions: diarrhoea in lambs (Harp et al. 1981), abortion (Long et al. 1978, Davies et al. 2001), epididymitis and orchitis (Ferreras et al. 2007) and chronic proliferative rhinitis (Meehan et al. 1992, Lacasta et al. 2012). Apart from chronic proliferative rhinitis, in the other clinical conditions the pathogenicity of *S. enterica* subsp*. diarizonae* is not clear and should be further studied.

S. enterica subsp*. diarizonae* is regularly isolated from faeces and tissues of gastrointestinal tract from apparently healthy lambs and adult sheep. The pathogen has been also detected in faeces and tissues of lambs that had died from diarrhoea, gaining attention as a potential causative agent. In the present thesis, (a) an outbreak of diarrhoea in newborn lambs caused by *S. enterica* subsp*. diarizonae* was described, (b) the implication of the organism in diarrhoeic syndrome of lambs has been further discussed and (c) the results of an experimental challenge of the pathogen in lambs have been presented.

Significance of *Salmonella enterica subsp. diarizonae* intestinal infections in lambs

Field outbreak of infection

S. enterica subsp *diarizonae* ser. 61:k:1,5,(7) was isolated from faecal samples of lambs during an outbreak of neonatal diarrhoea in a sheep farm in Thessaly, Greece. This is the first report of *S. enterica* subsp. *diarizonae* isolation from sheep in Greece.

This *Salmonella* serovar is often recovered from healthy sheep samples (Alvseike and Skjerve 2002, Lamas et al. 2018). However, the organism has been rarely implicated in clinical conditions (Davies et al. 2001, Alvseike and Skjerve 2002), with the exception of chronic proliferative rhinitis (Lacasta et al 2012, 2017). There are only few reports of isolation of the organism from lambs with diarrhoea or other disorders of the gastrointestinal tract (Harp et al. 1981, Sojka et al. 1983,

Davies et al. 2001). In all these studies, neither the organism had been isolated in pure culture as in the present one, nor an approach to achieve diagnosis of the problem had been followed.

The bacterium appears to be adapted to sheep, thus mainly remaining non-pathogenic. It has been recovered from faecal samples of healthy lambs (Bonke et al. 2012). In most cases, *S. enterica* subsp. *diarizonae* is considered to be ancillary to other pathogens.

During the outbreak, *S. enterica* subsp *diarizonae* ser. 61:k:1,5,(7) had been isolated from faecal samples from live animals and tissue samples harvested during post-mortem examination. Mostly, samples yielded the bacterium in pure culture and heavy growth, which suggests that it had inhibited growth of normal bacterial flora of the intestinal tract. The pathogen was also detected in tissue samples, which suggests that on this occasion, it was able to enter into the blood circulation and disseminate away from the intestine.

In this outbreak, *S. enterica* subsp. *diarizonae* ser. 61:k:1,5,(7) was isolated always in pure culture and consistently from a large number of lambs in the same flock. Hence, we consider this as the first description of *S. enterica* subsp. *diarizonae* ser. 61:k:1,5,(7) as causal agent of ovine neonatal diarrhoeic syndrome.

The main biochemical properties of the isolate(s) have been found to deviate from most isolates of *Salmonella* species. This isolate was a lactose fermenter and showed β-galactosidase production. In addition, tests for production of H_2S and both Lysine decarboxylase and D-sorbitol reactions were also positive. Lactose fermentation by the isolate was a significant deviation from results of *Salmonella* genus isolates. It is noteworthy that, in a recent study, Methner and Moog (2018) have reported that all isolates of *S. enterica* subsp. *diarizonae* ser. 61:k:1,5,(7) (n=74) recovered from faecal samples of sheep and evaluated were also lactose-fermenters. Unfortunately, no other relevant publication provides a detailed biochemical profile of isolates of the serovar, hence it is not possible to compare profile of the present isolate with those of other ones. Fermentation of lactose by *S. enterica* subsp. *diarizonae* ser. 61:k:1,5,(7) poses some difficulty in accurate differentiation – identification of the organism, as this result is different to most other *Salmonella* isolates. Based on that, procedures for identification of *Salmonella* to have been based on the principle of non-lactose fermentation.

In older references, it was found that isolates of *S. enterica* subsp. *diarizonae* ser. 61:k:1,5,(7) tested were, in general, susceptible to all antimicrobials tested against (Hall and Rowe 1992, Davies et al. 2001). Nevertheless, this trend progressively has started to change and isolates with resistance have now emerged (Bonke et al. 2012, Dargatz et al. 2015). The isolate recovered during the clinical outbreak described herein has shown resistance to some antimicrobials, which has forced

prescription, as per results of relevant tests, of a fluoroquinolone as the most appropriate agent for effective treatment.

Field investigation

In the field investigation, only lambs with diarrhoeic syndrome have been included as reference population, as objective was to evaluate the importance of *S. enterica* subsp. *diarizonae* as causal agent of the disease. The results have not indicated participation of the organism as causal agent of the entity.

It is interesting that athough the bacterium has been confirmed as part of the intestinal flora in healthy sheep, only rarely it has been isolated from cases of relevant disease and always, as discussed above, in mixed culture. The organism has nevertheless been isolated from faecal sampes of healthy lambs (Sandberg et al. 2002).

Samples from healthy lambs had not been collected during this study. Therefore, it could not have been possible to know whether there might have been presence of the organism in healthy animals. If that was the case, the lack of isolation indicates that, possibly, in cases of diarrhoeic syndrome overgrowth of these other pathogens had limited *S. enterica* subsp. *diarizonae* ser. 61:k:1,5,(7). Therefore, this bacterium, if present at all, had a limited role, if any, in the aetiology of diarrhoeic syndrome in these lambs.

Experimental infection

The results of the experimental work could not be aligned with those of the field work, both the study of the outbreak and the field investigation. One may suggest that experimental findings were ‘in-between’ those of the other two works.

After challenge, intestinal infection has been established in inoculated lambs. This was corroborated by consistent isolation of the challenge organism and definite evidence of inflammation during histopathological evaluation. Nevertheless, infection was mild and did not lead in fatalities as observed during the outbreak. In contrast however, to the findings of the field work, the organism was confirmed as an intestinal pathogen.

It is considered that possible differences in immune status of animals might have accounted for the different outcomes between outbreak findings, field work findings and results of experimental infection. The flock in which the outbreak had occurred, had previously been infected by *Bluetongue virus* during the large outbreak of the disease in Greece in the summer of 2014 (Kyriakis et al. 2015,

Vasileiou et al. 2016). This had been confimed by results of virological and immunological tests. Bluetongue can cause immunosupression in affected sheep; for example, Vasileiou et al. (2016) have reported increased incidence of mastitis after impediment of lymphocyte function (Vasileiou et al. 2016).

The finding of less frequent recoveries of the organism by microbiological techniques than frequency of detection of nucleid acid by molecular techniques lends support to this hypothesis. Likely, effective defences of the host eliminated the challenged organism, despite the high dose administered.

An incident of fatal gastrointestinal infection in newborn kids subsequently to bluetongue has been reported by Chatzopoulos et al. (2015) and Vasileiou et al. (2016). In that occasion, the disease was caused by *Citrobacter freundii*, another organism of limited clinical significance, but which has a confirmed pathogenicity in immunosuppressed hosts (Ambrosim et al. 2002; Galarneau et al. 2003).

After challenge, the isolate caused subclinical damage which was evident soon after inoculation. At the end, the challenge isolate had disseminated outside the gastriintestinal tract and was isolated from liver and gallbladder tissue samples. The findings are in contrast to a hypothesis by Katribe et al. (2009), who indicated that *S. enterica* subsp. *diarizonae* was limited in the intestinal tract; the findings are more allied to the results of Lacasta et al. (2017), who have also reported extra –intestinal (respiratory) infection of sheep with the pathogen.

The isolation of the organism from buccal swabs is of particular interest. Bacteria in the mouth of lambs could have originated from the inoculum or from regurgitation of gastric content. In field conditions, in which infection takes place orally the latter can apply. Isolation of *Salmonella* from milk samples of ewes is consistant with presence of the organism within the mouth and likely has been transferred during sucking by lambs. The possibility of bacteria transmission from lambs to suckling ewes has been shown by Fragkou et al. (2011).

In contrast to the findings during the outbreak, no gastrointestinal infection of dams of the inoculated lambs had been detected. Apart from the possibility of immunocompromise (as discussed above), increased bacterial numbers in the environment of the sheep farm, consequently to heavy excretion of the organism in faeces, might have also contributed.

Epilogue

Conclusions

The conclusions from the results of the present thesis are summarised herebelow.

(a) *S. enterica* subsp. *diarizonae* is an opportunistic gastrointestinal pathogen in lambs.

- The bacterium can cause a mild infection, with faecal shedding and definite histopathological changes in the gastrointestinal tract; the bacteria can be isolated from internal organs of infected animals.
- The bacterium can be a causal agent of the diarrhoeic syndrome of lambs.
- Faecal shedding of the bacterium is intermittent.
- Horizontal transmission of the bacterium between animals can be possible; it can be transmitted from lambs to ewes during sucking.
- Isolation of the organism from the milk of the dams of infected lambs is possible.

(b) Differences in severity of infection can be observed among animals and farms.

- Immunological condition of animals might be a determinant of the severity of disease.

Prospects

Suggestions for further research, in continuation of the present work, are as below.

- The evaluation of virulence determinants of various isolates of the organism, to evaluate possible significance in causing disease.
- Evaluation of the potential zoonotic implications, in view of the presence in milk.

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