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AN EPIDEMIOLOGIC APPRAISAL OF MILK TESTING FOR DIAGNOSIS AND CONTROL OF PARATUBERCULOSIS IN GREEK DAIRY SHEEP AND GOATS

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Summary

Chapter 1- Introduction

In the introductory chapter of this thesis, firstly, we present aspects of the distribution, immunology and pathogenesis of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection of ruminants. The reader should realize that the prolonged latent stage and incubation period hamper the early serological diagnosis of MAP infection. Then, some of the strengths and limitations of validation of tests with non gold standard methodology and Receiver Operating Characteristic curve analysis with Bayesian Mixture Models are presented. The reader should realize that the methodology is more powerful and well suited for evaluation of the serological diagnosis of MAP infection in dairy sheep and goats.

Chapter 2- Bayesian validation of a serum and milk ELISA for antibodies against Mycobacterium avium subspecies paratuberculosis in Greek dairy goats across lactation

The aim of the research presented in this chapter was to validate a commercial (IDEXX Pourquier, Montpellier, France) serum and milk indirect ELISA that detects antibodies against the MAP across lactation, in Greek dairy goats.

Each lactating goat was sampled at four consecutive times starting from kidding and covering the early, mid and late lactation stage. A total of 1268 paired milk/colostrum and blood samples were collected during the seven-month-long lactation period. Bayesian mixture models, which allow for the continuous interpretation of test results, were used to derive the distribution of the serum- and milk-ELISA response for the healthy and the MAP-infected individuals at each lactation stage. Both serum- and milk-ELISA, in all lactation stages, were of average and similar overall discriminatory ability as measured by the area under the curve. For each test, the lowest overlap between the distribution of the healthy and the MAP infected does was at late lactation. At this stage the area under the curve (AUC) was 0.89, 95% credible interval (0.70; 0.98) and 0.92

(0.74; 0.99) for the milk- and the serum-ELISA, respectively. Both tests had comparable sensitivities and specificities at the recommended cut-offs, across lactation. Lowering the cutoffs led to an increase in the sensitivities without serious loss in the specificities. In conclusion, the milk-ELISA can be as accurate as the serum-ELISA especially at the late lactation stage. Thus, it could serve as the diagnostic tool of choice, especially during the implementation of MAP control programs that require frequent testing, because milk sampling is a non-invasive, rapid and easy process. Finally, there is no need for lactation-stage specific selection to detect the disease as the prevalence is constant.

Chapter 3- Bayesian validation of a commercial milk and serum ELISA across lactation in dairy sheep

The aim of the research presented in this chapter was to evaluate a commercially available ELISA in sera and milk of a Greek dairy sheep flock. A total of 854 paired milk and blood samples were collected from ewes of a Greek flock and used to validate a commercial (IDEXX Pourquier, Montpellier, France) serum/milk ELISA against Mycobacterium avium subsp. paratuberculosis across lactation. We implemented Bayesian mixture models to derive the distribution of the responses of healthy and infected ewes. Both serum and milk ELISA had low to average overall discriminatory ability as measured by the area under the curves and comparable sensitivities and specificities at the recommended cut-offs. Lowering the cutoffs led to an increase in the sensitivities without serious loss in specificities.

Chapter 4- Flock-level factors associated with the risk of Mycobacterium avium subsp. paratuberculosis infection in Greek dairy goat flocks.

The aim of the research presented in this chapter was to conduct a cross-sectional study to identify flock-level risk factors for *Mycobacterium avium subsp. paratuberculosis* (MAP) infection, in Greek dairy goat flocks.

We collected 1599 milk samples from does that were at the last stage of lactation in 58 randomly selected dairy goat flocks, during May to September 2012. The collected samples were tested with a commercial milk ELISA (IdexxPourquier, Montpellier, France) and the results were

interpreted at a cut-off that optimized the accuracy of the diagnostic process. For the analysis of the data we used Bayesian models that adjusted for the imperfect Se and Sp of the milk-ELISA. Flock was included as a random effect. Does in flocks that used common water troughs and communal grazing grounds had 4.6 [95% Credible Interval (CI):1.5; 17.4] times higher odds of being MAP-infected compared to does in flocks that had no contact with other flocks. Does of flocks supplied with surface water from either streams or shallow wells had 3.7 (1.4; 10.4) times higher odds of being infected compared to those in flocks watered by underground and piped water sources. When kids were spending equal to or more than 10 hours per day with their dams they had 2.6 (1.1; 6.4) times higher odds of being MAP infected compared to kids that were separated from their dams for less than 10 hours per day. Finally, does in flocks that continuously used the same anti-parasitic compound had 2.2 (1.0; 4.6) times higher odds of MAP infection compared to those in flocks alternating anti-parasitic compounds. These results should be considered in the development of a nationwide future control program for caprine paratuberculosis in Greece.

Chapter 5- General discussion

In this chapter of the thesis the results of the research are summarized and discussed in relevance with existing knowledge and prospective follow up research activities.

Περίληψη

Κεφάλαιο 1- Εισαγωγή

Στο εισαγωγικό κεφάλαιο της παρούσας διατριβής, αρχικά παρουσιάζουμε πτυχές της κατανομής, της ανοσολογίας και της παθογένειας της λοίμωξης των μηρυκαστικών από το *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Ο αναγνώστης συνειδητοποιεί ότι η παρατεταμένη λανθάνουσα κατάσταση και η μεγάλη περίοδος επώασης αποτρέπουν την έγκαιρη ορολογική διάγνωση στα αρχικά στάδια της λοίμωξης από το MAP. Στη συνέχεια, παρουσιάζονται ορισμένα από τα πλεονεκτήματα και τα μειονεκτήματα των μεθόδων ανάλυσης της καμπύλης ROC, χωρίς διαγνωστική δοκιμή αναφοράς, με μικτά μοντέλα που επιλύονται κατά Bayes. Ο αναγνώστης αντιλαμβάνεται ότι η μεθοδολογία αυτή είναι η κατάλληλη για την εκτίμηση της διαγνωστικής αξιοπιστίας των ορολογικών διαγνωστικών δοκιμών, που ανιχνεύουν την λοίμωξη από MAP στα γαλακτοπαραγωγά πρόβατα και στις αίγες.

Κεφάλαιο 2- Εκτίμηση κατά Bayes της διαγνωστικής αξιοπιστίας μιας εμπορικής έμμεσης ELISA ενάντια του Mycobacterium avium subspecies paratuberculosis στους ορούς αίματος και γάλακτος εγχώριων αιγών σε διαφορετικά στάδια της γαλακτοπαραγωγικής περιόδου.

Ο σκοπός του κεφαλαίου αυτού της διατριβής είναι η παρουσίαση της μελέτης της εκτίμησης της διαγνωστικής αξιοπιστίας μιας εμπορικά διαθέσιμης έμμεσης (IDEXX Pourquier, Montpellier, France) ELISA που ανιχνεύει αντισώματα κατά του MAP σε δείγματα ορών αίματος και γάλακτος ελληνικών αιγών για διαφορετικά στάδια της γαλακτοπαραγωγικής περιόδου.

Από ένα κοπάδι αιγών γαλακτοπαραγωγικής κατεύθυνσης συλλέχθηκαν συνολικά 1268 δείγματα ζευγών αίματος και γάλακτος ή πρωτογάλακτος. Τα ζεύγη δειγμάτων συλλέχθηκαν από κάθε αίγα του κοπαδιού τέσσερις επαναλαμβανόμενες φορές, ξεκινώντας κατά τη γαλουχία και συνεχίζοντας, καλύπτοντας την αρχή, το μέσο και το τέλος της γαλακτοπαραγωγικής περιόδου. Η ανάλυση έγινε με τη χρήση μικτών μοντέλων, που επιλύθηκαν κατά Bayes, τα οποία επέτρεπαν την εκτίμηση των αποτελεσμάτων σε συνεχή κλίμακα. Με τα μοντέλα προβλέφθηκαν οι κατανομές των αποτελεσμάτων της ELISA τόσο

στους ορούς αίματος όσο και στους ορούς γάλακτος από υγιείς και μολυσμένες με MAP αίγες για τα τέσσερα στάδια της γαλακτοπαραγωγικής περιόδου.

Τόσο στους ορούς αίματος όσο και στους ορούς γάλακτος, σε κάθε στάδιο της γαλακτοπαραγωγικής περιόδου, η ELISA εκτιμήθηκε ως διαγνωστική δοκιμή μέτριας διακριτικής ικανότητας, όπως αυτό προέκυψε από το εμβαδόν της καμπύλης ROC. Για κάθε δείγμα, η μικρότερη αλληλοεπικάλυψη των κατανομών των υγιών και των μολυσμένων αιγών με MAP βρέθηκε στο τελικό στάδιο της περιόδου. Σε αυτό το στάδιο, η διάμεση τιμή του εμβαδού κάτω από την καμπύλη ROC της ELISA βρέθηκε να είναι 0,89 με 95% διάστημα αξιοπιστίας (0,70-0,98) και 0,92 (0,74-0.99) στους ορούς γάλακτος και αίματος, αντίστοιχα. Και οι δύο διαγνωστικές δοκιμές ELISA στους ορούς αίματος και γάλακτος για τα διαφορετικά στάδια της γαλακτοπαραγωγής, είχαν συγκριτικά παρόμοιες ευαισθησίες και ειδικότητες. Επίσης, φάνηκε ότι μειώνοντας το κρίσιμο σημείο διάκρισης αυξήθηκε η ευαισθησία χωρίς σοβαρές απώλειες σε ειδικότητα.

Εν κατακλείδι, η ELISA στους ορούς γάλακτος είναι εξίσου αξιόπιστη με την ELISA στους ορούς αίματος ειδικά κατά το τελευταίο στάδιο της γαλακτοπαραγωγής. Επομένως, θα μπορούσε να χρησιμοποιηθεί ως διαγνωστικό εργαλείο εκλογής, ειδικά κατά την εφαρμογή προγραμμάτων ελέγχου της λοίμωξης από MAP που απαιτούν επαναλαμβανόμενες δειγματοληψίες, καθώς το γάλα συλλέγεται ευκολότερα σε σχέση με το αίμα, με μη επεμβατικό τρόπο και γρήγορα. Τέλος, δεν υπάρχει ανάγκη για εκτίμηση ειδικού σταδίου της γαλακτοπαραγωγής κρίσιμου σημείου διάκρισης, καθώς το ποσοστό προσβολής παρέμενε σταθερό.

Κεφάλαιο 3- Εκτίμηση κατά Bayes μιας εμπορικά διαθέσιμης ELISA σε δείγματα γάλακτος και αίματος για τα διαφορετικά στάδια της γαλακτοπαραγωγικής περιόδου προβάτων.

Ο σκοπός αυτής της μελέτης που παρουσιάζεται στο κεφάλαιο αυτό ήταν η εκτίμηση της διαγνωστικής αξιοπιστίας μιας εμπορικά διαθέσιμης ELISA (IDEXX Pourquier, Montpellier, France) σε δείγματα ορών αίματος και γάλακτος. Συλλέχτηκαν συνολικά 854 ζεύγη δειγμάτων αίματος και γάλακτος από τις προβατίνες ενός εγχώριου κοπαδιού προβάτων με σκοπό την εκτίμηση μιας εμπορικής ELISA ενάντια του *Mycobacterium avium* subsp. *Paratuberculosis* για

κάθε στάδιο της γαλακτοπαραγωγής. Εφαρμόστηκαν μικτά μοντέλα κατά Bayes για να προβλεφθούν οι κατανομές των αποτελεσμάτων της ELISA στις υγιείς και στις μολυσμένες από MAP προβατίνες. Τόσο η ELISA στο αίμα όσο και στο γάλα είχαν χαμηλή έως μέτρια συνολική διακριτική ικανότητα, όπως αυτή μετρήθηκε με την περιοχή του εμβαδού κάτω από την καμπύλη ROC και είχαν στατιστικά σημαντικά όμοιες ευαισθησίες και ειδικότητες στα προτεινόμενα από την εταιρεία κρίσιμα σημεία διάκρισης. Μειώνοντας το κρίσιμο σημείο διάκρισης αυξήθηκαν οι ευαισθησίες χωρίς σημαντική μείωση στις ειδικότητες.

Κεφάλαιο 4- Παράγοντες επικινδυνότητας σε επίπεδο κοπαδιού που συσχετίζονται με την πιθανότητα εμφάνισης λοίμωξης από το Mycobacterium avium subsp. paratuberculosis (MAP) σε ελληνικά κοπάδια αιγών γαλακτοπαραγωγής.

Ο σκοπός της έρευνας που παρουσιάζεται στο κεφάλαιο αυτό ήταν η διεξαγωγή μιας μελέτης χρονικού σημείου, με σκοπό την ανίχνευση παραγόντων επικινδυνότητας σε επίπεδο κοπαδιού, που αυξάνουν την πιθανότητα εμφάνισης λοίμωξης από το *Mycobacterium avium* subsp. *paratuberculosis* (MAP) στα εγχώρια κοπάδια αιγών γαλακτοπαραγωγικής κατεύθυνσης.

Από το Μάιο έως τον Σεπτέμβριο 2012 συνολικά συλλέχθηκαν 1599 δείγματα γάλακτος από αίγες 58 τυχαία επιλεγμένων κοπαδιών στο τελικό στάδιο της γαλακτοπαραγωγικής τους περιόδου. Τα δείγματα που συλλέχθηκαν εξετάστηκαν με μια εμπορική ELISA (IdexxPourquier, Montpellier, France) και τα αποτελέσματα αναλύθηκαν χρησιμοποιώντας το κρίσιμο σημείο διάκρισης που βελτιστοποιούσε την ακρίβεια της διαγνωστικής δοκιμής. Για την ανάλυση των δεδομένων χρησιμοποιήθηκε ένα μοντέλο κατά Bayes, διορθώνοντας για την μη τέλεια ευαισθησία και ειδικότητα της ELISA στο γάλα. Το επίπεδο του κοπαδιού θεωρήθηκε τυχαία επίδραση.

Οι αίγες των κοπαδιών που χρησιμοποιούν κοινούς βοσκοτόπους και ποτίστρες με άλλα κοπάδια είχαν 4,6 φορές [95% Διάστημα Αξιοπιστίας (ΔΑ):1,5-17,4] μεγαλύτερη πιθανότητα να εμφανίσουν λοίμωξη από MAP σε σχέση με τις αίγες των κοπαδιών που δεν ήρθαν σε επαφή με άλλα κοπάδια. Οι αίγες των κοπαδιών που ποτίζονταν από επιφανειακές πηγές νερού είχαν 3,7 (1,4-10,4) φορές μεγαλύτερη πιθανότητα να είναι μολυσμένες σε σχέση με τις αίγες των κοπαδιών στις οποίες η παροχή του νερού προερχόταν από μη επιφανειακές πηγές (ύδρευση, γεώτρηση). Όταν τα ερίφια των αιγών των κοπαδιών σταβλίζονταν για περισσότερες από 10

ώρες με τις μητέρες τους αυτές είχαν 2,6 (1,1-6,4) φορές μεγαλύτερη πιθανότητα εμφάνισης λοίμωξης από MAP σε σχέση με τις αίγες που τα ερίφιά τους σταβλίζονταν λιγότερο από 10 ώρες την ημέρα με αυτές. Τέλος, διαπιστώθηκε ότι οι αίγες των κοπαδιών, στις οποίες χορηγούνταν τα ανθελμινθικά σκευάσματα χωρίς κυκλικές εναλλαγές είχαν 2,2 (1,0-4,6) φορές μεγαλύτερη πιθανότητα να είναι μολυσμένα από MAP σε σχέση με τις αίγες των κοπαδιών που έκαναν εναλλαγές των ανθελμινθικών ουσιών. Τα αποτελέσματα της έρευνας αυτής θα μπορούσαν να χρησιμοποιηθούν σε μελλοντικό πρόγραμμα ελέγχου της παραφυματίωσης των αιγών στην Ελλάδα.

Κεφάλαιο 5- Συζήτηση

Σε αυτό το κεφάλαιο της διατριβής συνοψίζονται τα αποτελέσματα της έρευνας και αναπτύσσονται σε σχέση με την υπάρχουσα γνώση και τις μελλοντικές προοπτικές έρευνας.

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Chapter 1

1. Introduction to paratuberculosis

1.1.1 Etiology and distribution of paratuberculosis

Paratuberculosis is a chronic intestinal disease mainly of ruminants, with worldwide distribution, which was first ''officially'' described in 1895 (Johne and Frothingham, 1895), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Johne and Frothingham found organisms in granulomatous lesions in the intestines of affected cattle that stained acid-fast, indicating some type of mycobacterial organism. Later, MAP was identified by Twort and Ingram (1912). MAP is a gram positive acid-fast bacterium dependant on the presence of iron from the host to develop (Kennedy and Benedictus, 2001). MAP produces reductase an enzyme which mobilizes iron from the host (Homuth et al., 1998). In vitro, MAP depends on the presence of mycobactin to grow. However, some MAP strains grow in vitro without addition of mycobactin (Lamont et al., 2013). Based on molecular and cultural characteristics two major groups of MAP strains are distinguished the cattle (C) or type I and the sheep (S) or type II (Collins et al., 1990; Whittington et al., 1998; Pavlik et al., 1999; Stevenson et al., 2002). The type I affects mainly cattle and goats and the type II affects mainly sheep. However, a direct correlation between strain type and the host species does not exist (Stevenson et al., 2002). The type I strains have been isolated from goats and sheep that were on the same pasture with cattle (Riemann et al., 1979; Ris et al., 1988; Greig et al., 1999; Beard et al., 2001, Stevenson et al, 2002). In Greece, both MAP types were recovered from sheep and goats of mixed flocks (Florou et al., 2007). MAP has a lipid-rich cell wall which facilitates its survival and persistence in the environment. It is able to survive exposure to high temperatures and the use of detergents (Kennedy and Benedictus, 2001). It may persist in the water for nine months (Lovel et al, 1954), in manure for eight months and in slurry for a month (Larsen et al., 1956). It survives longer on acid than alkaline soil (Kopecky, 1977).

MAP infection has a worldwide distribution mainly, but not only, affecting farmed and wild ruminants. Especially in sheep and goats the infection has been reported in many countries in the southern, as in Australia, New Zealand and South Africa, and the northern hemisphere, particularly in Great Britain, the Mediterranean countries including Greece, Turkey, Spain,

Portugal, Morocco, France and in Norway, Switzerland, Croatia, Canada, the USA, and Chile (Barkema et al., 2010; Benazzi et al., 1995; Djønne, 2010; Hailat et al., 2010). In Greece, paratuberculosis was first reported in 1968, when typical gross lesions were found and MAP was cultured from diseased goats (Leontides et al., 1975). Today, the majority of Greek sheep and goat flocks are endemically infected with MAP (Kostoulas et al., 2006a; Ikonomopoulos et al., 2007; Dimareli-Malli et al., 2013). Florou et al. (2008) isolated MAP from wildlife species co-habiting the sheds and/or the grazing grounds of Greek sheep and goat flocks. Furthermore, MAP was cultured and MAP DNA was recovered from traditional cheeses made from sheep and goat milk (Ikonomopoulos et al., 2005).

1.1.2 MAP infection and pathogenesis of paratuberculosis

MAP infection mostly results from the fecal-oral exposure of a sensitive animal. The fecal-oral exposure may occur from ingestion of MAP through ingestion of milk from a fecally contaminated teat, or exposure to manure contaminated pasture, water, supplements or hay (Windsor and Whittington, 2010). Infection may also occur through drinking of MAP contaminated colostrum or milk, because MAP is excreted in colostrum and milk of infected sheep and goats (Lambeth et al., 2004; Nebbia et al., 2006). Finally, intra-uterine MAP infection of fetuses of clinically affected dams is also now well described (Lambeth et al., 2004; Whittington and Windsor, 2009).

After ingestion, MAP is transported through the intestinal wall via the M cells that serve as portals of MAP entrance in the gut-associated lymphoid system (Momotani et al., 1988; Stabel, 2000). Then it is taken up by macrophages or dentritic cells. In the macrophages, it may be degraded or stay intact and proliferate. When MAP is presented to the T cells induces cellular or humoral immune response. At the early stages of infection the protective cellular immune response is produced: A Th1 population of CD4+ cells produce cytokines, such as interferon-gamma (IFN-γ), that activate the cellular immune response contributing to the activation of the macrophages and control of MAP proliferation. Later, a shift to the humoral immune response occurs that acts unprotectively against MAP (Stabel, 2000): the Th2 cells produce cytokines

important in the unprotective humoral immune response (Sigurðardóttir et al., 2004; Stabel, 2006).

Despite exhaustive efforts, until today we do not understand all steps of MAP infection and proliferation (Momotani and Eda, 2014). The mechanisms that onset the shift of the immune response could be: the prolonged exposure of the T-cells to the antigen released from the macrophages, the development or not of the antigen specific regulatory cell populations and genetic factors of the host (Coussens, 2004). Lights on the pathogenesis of the disease were shed when the genome sequence of MAP K-10 was completed (Li et al., 2005) and revealed the virulence factors and regulatory elements encoded in this genome. Myriad of regulatory proteins required for cell adaptation to survive under wide spectrum of microenvironments are present in the MAP genome (Talaat, 2014).

In conclusion, MAP infection develops slowly in several stages. After MAP has been transmitted, the animal may escape infection or get infected but control MAP spread in its gut. Clearance may occur or some inactivated macrophages may remain in granulomas. For unknown reasons, at unpredicted time point, stimulation of the humoral immune response occurs and begins the production of IgG antibodies. MAP infection progresses as the humoral immune response is non-protective and MAP spreads in the host's gut. This results in increasing granuloma formation and extreme thickening of the intestines. The typical clinical symptoms of paratuberculosis become apparent: weight loss, diarrhea, losses in productivity and eventually death. Although the above description of the disease is the well-known text book variant the reality in animals and populations is more complex. Only a minority of infected animals will progress to the typical clinical stage described above. Some animals do not get infected or may overcome and clear the infection, while the majority of infected animals will be in a protracted latent stage, they remain so during their lifetime while shedding and spreading MAP in their environment.

1.2 Serology for diagnosis of MAP infection

Although there are arguments in favor of organism detecting over the use of serological assays, such as better Sensitivity (Se) and Specificity (Sp) (Collins et al., 2005), there are several practical reasons to prefer the use of serological assays as the basis for diagnosis of MAP infection in the design of paratuberculosis control programs. Serological assays are usually cheap, fast and, especially ELISA, can be automated. Their principal limitation is that they detect antibodies which are produced at the later stage of MAP infection (Collins, 1996; Stabel, 1997).

Among the available serological methods the ELISA is by far the most frequently used. Since the first introduction of the ELISA method (Engvall and Perlmann,1971) a wide variety of antigens have been used in a large number of different ELISA's for detection of MAP infection (Griffin et al, 2005). These antigens were "crude antigens" and often resulted in false positive reactions (Bakker, 2014). A major breakthrough was the introduction of the absorbed ELISA by Yokomizo et al. (1985) were the serum to be tested was pre-absorbed with an extract of *Mycobacterium phlei*, a rapid growing mycobacterial species. This procedure resulted in increased Sp of the ELISA (Yokomizo et al., 1985).

The absorbed ELISA (thereafter "ELISA") may detect antibodies in serum or milk. The use of milk may be preferred over serum because sampling is cheaper since it is usually done by farmers repeatedly, for milk-testing for a number of other reasons. Therefore, if one considers the development of a national control program against MAP-infection in a country with a large number of small ruminants reared all over its territory (National Agricultural Research Foundation), ELISA milk testing is the least expensive choice. However, because the ELISA is not a perfect diagnostic test, its prudent use requires prior acknowledgment of its limitations (Nielsen, 2009). Unfortunately, before the research compiled in this thesis, there were no available estimates of the ELISA diagnostic validity in dairy sheep and goats milk samples.

The diagnostic validity of a test is described by the Se and Sp, which are considered as innate characteristics of the test for a defined reference population at a specific cut-off point. Although they are considered relatively stable measurements, Se and Sp can vary. This variation is mainly attributable to differences among the reference populations and sampling strategies that have been used for the validation procedure (Greiner and Gardner, 2000). Greiner and Gardner (2000) showed that estimates for Se and Sp may vary among populations and/or subpopulations of animals, conditional on the distribution of influential covariates. For example, they may be related to differences in severity of lesions and host characteristics (Sergeant et al.,

2003). When a test is evaluated, one should consider the specific use it is intended for. If not, the result of the evaluation may be biased, either because of problems with establishing the true infection status or because the test detects another aspect of the infection than originally intended (Nielsen et al., 2011). For MAP serology, several authors indicated the need for species- (Kostoulas et al., 2006a), strain- (Florou et al., 2009), lactation stage- (Nielsen et al., 2002a) and target condition- (Nielsen et al, 2007) specific evaluation. Recently, in order to improve the quality of reporting test validation for paratuberculosis an expert-derived list of items was developed [Standards for Reporting of Animal Diagnostic Accuracy Studies for paratuberculosis (STRADAS-paraTB)](Gardner et al., 2011).

Reported estimates of Se usually were based on cases of paratuberculosis confirmed by pathological changes combined with a positive culture from feacal or tissue samples in sheep (Hilbink et al., 1994; Dubash et al., 1995; Clark et al., 1996; Hope et al., 2001; Sergeant et al., 2003), goats (Molina et al., 1991; Rajukumar et al., 2001; Salgado et al., 2007) and mixed sheep and goat flocks (Munjal et al., 2004). Erroneously, the faecal culture (FC) was regarded as gold-standard (GS) method for determining MAP infection (National Research Council, 2003). FC may not detect MAP at the early stages of infection (Whitlock and Buergelt, 1996) and at the late stages uneven shedding of bacteria can occur (Whittington and Sergeant, 2001). Also, MAP infection often may be missed by histopathological examination, either because the pathologists were not perfect or because the bacteria had not yet caused detectable pathological changes (Whittington et al., 1999). Hence, such evaluations did not include all latent cases of infection (Nielsen et al., 2002b) and the published Se and Sp estimates were in reality relative Se and Sp estimates to imperfect diagnostic tests.

Alternative non-gold standard (NGS) methods with the use of latent class models have been applied. In general, in latent class models two or more diagnostic tests are applied in one or more populations and none of the tests is perfect (Kostoulas et al., 2006a). The disease status is designed latent -existing but not present or evident or realized- and the models create their own probabilistic definition of disease, in our case MAP infection. For binary outcome test data, Hui and Walter (1980) used maximum likelihood methods to estimate Se and Sp when a GS test was not available. Bayesian methodology has also been applied for the model proposed by Hui and Walter (Johnson et al, 2001; Joseph et al., 1995; Georgiadis et al., 2003; Dendukuri and Joseph 2001; Black and Graig 2002) resulting in an increased use of Bayesian modeling for

estimation of test accuracy in veterinary medicine (Gardner, 2002). Diagnostic-test evaluation is particularly suited to the Bayesian framework because prior scientific information about the Se and Sp of the tests and prior information about the prevalences of the sampled populations can be incorporated (Bransum et al., 2005). Kostoulas et al., 2006a estimated the Se and Sp of a serum ELISA in dairy sheep and goats with latent class models in a Bayesian framework. They reported medians (Credible Intervals) of Se and Sp as 63% (42; 93%), 95% (90; 98%), in goats and 37% (10; 80%), 97% (93; 99%), in sheep.

1.3 ROC Analysis with the Bayesian Mixture Models

For tests with a continuous outcome, like the ELISA, the analysis of the Receiver Operating Characteristic (ROC) curve is important for the evaluation of the test performance and comparison with other tests over the entire spectrum of possible outcomes, as well as for the optimization of the cutoff selection process for different test applications (Greiner et al., 2000). The ROC curve depicts the relationship between pairs of true positive rates (Se), on the vertical axis, and false-positive rates (1 - Sp), on the horizontal axis, for all possible cutoffs, thus measuring the overall discriminatory power of the test. The perfect test, which discriminates perfectly between MAP infected and healthy animals, generates a curve that coincides with the left side and top of the plot. A nugatory test, in contrast, would produce a straight-line plot, from bottom left to top right. Thus, tests with ROC curves furthest into the top left corner are better tests. The Area Under the Curve (AUC) is a global measure of a test's performance. This area equals the probability that a random individual with the infection has a higher value of the test outcome than a random individual without the infection. A perfect test thus yields an AUC of 1, whereas an uninformative test yields a value of 0.5.

Traditionally, ROC analysis assumes the existence of a GS reference test that has perfect Se and Sp. However, since the first introduction of the NGS methodology applied on binary data by Hui and Walter (1980) many others have developed NGS methods for ROC analysis with the use of maximum likelihood inference applied on ordinal or continuous test outcomes (Henkelman 1990; Beiden et al., 2000; Nielsen, 2002b; Hall and Zou, 2003). Later, Bayesian

methodology for non-parametric estimation of ROC curves in the absence of a GS, as well as parametric estimation, where the test values (or the transformed test values) of the non-infected and infected individuals are assumed to follow a normal distribution was developed (Choi et al., 2006). Mmore recently, Jafarzadeh et al. (2010), developed a Bayesian mixture model for continuous test values with limit on detection based on the assumption of normality or gamma distributed data.

The data from a serological assay derived from a population of healthy and MAP infected individuals corresponds to a mixture distribution. In such instances, mixture models are used to discriminate between the different subpopulations. Under a mixture model an unobserved or missing indicator variable – the latent variable - is introduced. The latent variable is categorical, but the observed variables may be either categorical or continuous. Thus the observed variables are modeled conditionally on the latent variable (Gelman et al., 2004). Although the mixture models are appealing in the application of NGS methodology for ROC curve analysis, the problem of identifiability may arise. Identifiability refers to the existence of a unique characterization for any one of the models lying in the population (McLachlan and Peel, 2000) .If a model is not identifiable the estimation procedure may not be well-defined and asymptotic theory may not hold. To assure identifiability, we try to set distinct representations of the models and/or use relevant and scientifically justified prior information. Bayesian inference allows for the incorporation of informative priors so that prior knowledge or results of previous studies can be used to inform the current model (Gardner, 2002). However, these should subsequently be subject to sensitivity analysis to assess the effect of the specified priors on the estimated parameters (Njtoufraz, 2009).

1.4 Background and objectives

Only, few published studies were based on latent class models to evaluate the performance of the serum ELISA in sheep and goats (Kostoulas et al., 2006a and b). The characteristics of the milk ELISA in goats have been estimated with the use of reference tests with imperfect Se and Sp (Salgado et al., 2005; Salgado et al., 2007; Kumar et al., 2008). There are no studies evaluating the milk ELISA in dairy sheep and goats with NGS methods. In addition, a study in dairy cattle (Nielsen et al., 2002a) revealed that the milk antibody trend varies across

lactation indicating that there might be a need for evaluation across lactation. Greece has the largest goat herd in the EU accounting for around 50% of the EU total and is self-sufficient in goat-meat (National Agricultural Research Foundation). The Greek national herd comprises of approximately 4 million goats and 10 million sheep, which are reared primarily for milk production (Food and Agriculture Organization of the United Nations Statistics Division). The main reason why there are so many sheep and goats in Greece is because there is a strong tradition of cheese consumption in the Greek gastronomy; cheese is not a food supplement, it is food. Contrary to its European counterparts of France, Italy and Spain, Greeks consume cheese at all times, i.e. for breakfast, lunch, dinner, alone or with other food, having the highest consumption in EU of 23 kg per person per year. A plethora of protected destination of origin (e.g. feta) or protected geographical indication cheeses of Greece are dependent on the production of sheep and goat milk. Many of these cheeses are exported to other EU-countries, the USA and Australia. In a study on the prevalence of MAP in retail feta cheese (produced from sheep and goat milk) the authors reported 50% (21⁄42) and 4.7% (2⁄42) PCR- and culture-positivity, respectively, for MAP (Ikonomopoulos et al., 2005). A potential zoonotic link between MAP and human inflammatory bowel diseases including Crohn's disease has been suggested but remains unclear (Over et al., 2011). If MAP is confirmed as a zoonotic pathogen, public confidence in products of the Greek small ruminant industry is very likely to decline. In other countries exporting products mainly from dairy cows national control programs to control MAP infection have been developed and implemented. For example in the Netherlands, milk ELISA is used for whole-herd testing aiming at reducing the concentration of MAP in bulk milk (Weber, 2008). In the United States the Voluntary Johne's Disease Herd Status Program (Bulaga, 1998) aims at herd-manager education, establishment of management strategies to reduce the spread of MAP and herd classification based on diagnostic test results (Carter, 2007). In Denmark, the program is run as a risk-based control programme: All enrolled herds are tested four times per year using an antibody test, and cows are classified into high- and low-risk cows; specific measures are established to reduce potential MAP transmission from the former cows (Nielsen, 2009b).

Prerequisite to the development and implementation of these programs were the evaluation of the validity of the diagnostic tests applied as "backbones" of the programs and the identification of risk factors for within and among herds spread of MAP. The research compiled

in this thesis generated relevant information for the development of a Greek national control program in dairy sheep and goats. The primary objective of the thesis was the validation of the milk ELISA as a tool to monitor MAP infection in Greek dairy sheep and goats. The milk ELISA is a relatively fast and cheap diagnostic method that can be automated. Furthermore, the cost of sample collection is minimized because farmers are repeatedly collecting milk for testing for other purposes. The test was validated across lactation by the use of Bayesian mixture models, applying NGS methodology, separately in dairy sheep and goats. It was shown that the milk ELISA in dairy goats performed equally well with the serum ELISA and had good overall discriminatory ability. However, its discriminatory ability in dairy sheep was inferior. Therefore, the ELISA was subsequently employed as the diagnostic tool to identify managerial factors associated with the risk of MAP infection in Greek dairy goat flocks.

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Chapter 2

Bayesian validation of a serum and milk ELISA for antibodies against Mycobacterium avium subspecies paratuberculosis in Greek dairy goats across lactation

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2.1 Abstract

We validated a commercial (IDEXX Pourquier, Montpellier, France) serum and milk indirect ELISA that detects antibodies against the *Mycobacterium avium* subsp*. paratuberculosis* (MAP) across lactation, in Greek dairy goats. Each lactating goat was sampled at four consecutive times starting from kidding and covering the early, mid and late lactation stage. A total of 1268 paired milk/colostrum and blood samples were collected during the seven-month-long lactation period. Bayesian latent class models, which allow for the continuous interpretation of test results, were used to derive the distribution of the serum- and milk-ELISA response for the healthy and the MAP-infected individuals at each lactation stage. Both serum- and milk-ELISA, in all lactation stages, were of average and similar overall discriminatory ability as measured by the area under the curve. For each test, the lowest overlap between the distribution of the healthy and the MAP infected does was at late lactation. At this stage the area under the curve was 0.89, 95% credible interval (0.70; 0.98) and 0.92 (0.74; 0.99) for the milk- and the serum-ELISA, respectively. Both tests had comparable sensitivities and specificities at the recommended cut-offs, across lactation. Lowering the cutoffs led to an increase in the sensitivities without serious loss in the specificities. In conclusion, the milk-ELISA can be as accurate as the serum-ELISA especially at the late lactation stage. Thus, it could serve as the diagnostic tool of choice, especially during the implementation of MAP control programs that require frequent testing, because milk sampling is a non-invasive, rapid and easy process. Finally, there is no need for lactation-stage specific selection to detect the disease as the prevalence is constant.

Key words: Bayesian latent class model, paratuberculosis, serum and milk ELISA, dairy goat

2.2 Introduction

Paratuberculosis, which is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), induces a chronic intestinal infection in cattle, sheep, goats and other ruminants. The disease decreases productivity, leads to suboptimal productive life and, thus, causes substantial economic losses to the farming industry (Clarke et al., 1997). Goats can become persistent faecal shedders about 1 year post-infection without any clinical signs of paratuberculosis (Stores et al., 2001) during a long latent subclinical phase. Early clinical signs of the disease include progressive wasting and decrease in milk production that are followed by manifestations of advanced clinical disease: flaky skin, poor hair coat, progressive emaciation, dehydration, anemia with submandibular edema, depression and diarrhea (Stehman et al., 1996). Commonly used diagnostic tests, such as the ELISA and the fecal culture, are of low sensitivity (Se) for identifying infected individuals during the early latent infection stage (Bakker et al., 2000).

An impediment to surveillance and control of paratuberculosis is the cost of testing, particularly for small ruminant industries, because of the low economic value of each animal (Salgado et al., 2007). Specifically, diagnosis of paratuberculosis by fecal culture is slow, laborious and expensive. In contrast, serum ELISA is quick and automated but sample collection may be laborious and increase the cost of surveillance. Compared to serum-ELISA, the milk-ELISA has the advantage of easy sample collection. Its validation could make paratuberculosis testing more affordable and more widely applicable as a useful tool for the management of this disease by dairy goat farmers.

To validate a diagnostic test, a reference test is needed to ascertain the true disease status for the healthy and the infected populations. Because a perfect reference test does not exist, many authors have acknowledged the need for latent class methods (Branscum et al., 2005; Kostoulas et al., 2006a and b; Wang et al., 2007) that account for all latently infected individuals in order to obtain valid estimates of the validity of diagnostic tests. Recently, Bayesian latent class models which do not require dichotomization of the test outcomes and apply to the actual continuous test results have been proposed (Choi et al., 2006; Jafarzadeh et al., 2010; Wang et al., 2011). The advantage of this approach is that the actual distributions of

the healthy and the infected populations can be derived. Thus, the continuous interpretation of test results is feasible, avoiding simple definitions of the MAP infection which can be difficult and even misleading due to the chronicity of the infection (Toft et al., 2005). For example, dichotomization of the ELISA results leads in loss of valuable information conveyed in the test by disregarding the fact that all positives aren't equal.

In dairy cattle, the milk-ELISA has been evaluated across lactation (Nielsen et. al, 2002b), compared to serum-ELISA without (Hendrick et al., 2005; Lombard et al., 2006; Kennedy and Benedictus, 2001) and with models that allow continuous interpretation of the results (Kostoulas et al., 2013). However, we cannot extrapolate results from the dairy cattle to dairy goats. Authors have indicated the need for species- (Kostoulas et al., 2006), strain- (Florou et al., 2009), target condition- (Nielsen et al, 2007) and lactation stage- (Nielsen et al., 2002b) specific evaluation of MAP diagnostics. In dairy goats, few studies evaluated the milk-ELISA (Kumar et al., 2008; Salgado et al., 2005; Salgado et al., 2007) but not across lactation, without the use of latent class models and continuous results interpretation. Therefore, the objective of this study was to assess and compare the overall diagnostic validity of a commercial ELISA kit between milk and serum samples, at different lactation stages, in Greek dairy goats. The latent-class analyses were done in a Bayesian framework.

2.3 Materials and methods

2.3.1 Study population and sampling scheme

A flock with 300 dairy goats was selected for the study. The flock had a history of clinical paratuberculosis and was unvaccinated against MAP. The does were of the domestic breed or their crosses with Alpine breed. The age of the does ranged from one to eight years old (median four years).The animals were kept under semi-intensive management for milk production, which was the primary breeding goal. The farmers selected replacements among the daughters of high-yielding does. The males bought into the flocks originated from high-yielding animals from other flocks. The animals grazed on pasture throughout most of the year and were additionally

fed concentrates. They spent most of the day outside and were moved into the shed during the night. They were mated to bucks, in an unsupervised manner, in June– September and delivered during December and March of the following year. The kids were weaned 45–60 days after birth; subsequently the does were hand-milked. Milking was ceased abruptly when the stockman felt that the milk yield was so reduced that it did not pay off the milking routine and the extra feeding.

The animals were followed up from December 2008 to March 2010. We collected a total of 1,268 milk/colostrum and blood samples during the seven-month-long lactation period. Each lactating goat was sampled at four consecutive times starting from kidding and covering the early, mid and late lactation stage. Sampling of the does by lactation stage and date is shown in Figure 2.1.

2.3.2 Diagnostic Tests

Collected milk and colostrum samples were centrifuged, skimmed (-8^{o}C, 1600 g/20min) and stored at -21^{o}C, until testing. Sera were tested using a commercial indirect ELISA kit (IDEXX Pourquier, Montpellier, France) according to the manufacturer's protocol. Skimmed colostrum and milk samples were tested by the same ELISA using the proposed manufacture's protocol for bovine milk (Salgado et al. 2007). The paired sera and milk samples were tested simultaneously in order to avoid in-plate and in-day variability (Nielsen et al., 2002a). The recorded optical densities (**OD**) were transformed to the Sample to Positive (**S/P**) ratio, which were kept on a continuous scale for further analysis (Toft et al., 2005).

2.3.4 Statistical Analyses

We implemented a Bayesian mixture modeling approach in order to predict the distribution of the serum- and milk-ELISA response by infection status (healthy or diseased) separately for each lactation stage.

Bayesian Mixture Model. The proposed model determines the distribution of the continuous serum- and milk- ELISA response by infection status and lactation stage, adjusting for

the likely correlation of the OD measurements within animal and lactation stage. For each infection status we assume that either the original continuous test responses are normally distributed or can be transformed to normality using appropriate methods such as the log-transformation (Nielsen et al., 2007; Toft et. al, 2005). Let Y_{ij} denote the log-transformed ELISA response of the i[th] doe at the j[th] lactation stage, with $j = 1,\ldots,4$ ($j = 5,\ldots,8$) corresponding to kidding, early, mid and late lactation stage for the serum- (milk-) ELISA. Also, let D_{ij} be the latent data that represents the unknown true disease status of each doe at each lactation stage, with $D_{ij} = 0$ for the healthy and $D_{ij} = 1$ for the diseased individuals. The Y_{ij} follow a mixture multivariate normal distribution, with two mixture components:

$$(Y_{ij} \,|\, D_{ij}) \sim \varphi(\cdot \,|\, \mu_{j1}, \Sigma_{jj1})^{D_{ij}} \varphi(\cdot \,|\, \mu_{j0}, \Sigma_{jj0})^{1-D_{ij}}$$

$$D_{ij} \sim Bernoulli(\pi_j)$$

$$\mathbf{\Sigma_{jj0}} = \begin{pmatrix} \sigma^2_{110} & \cdots & \sigma_{1j0} \\ \vdots & \ddots & \vdots \\ \sigma_{j10} & \cdots & \sigma^2_{jj0} \end{pmatrix}$$

$$\mathbf{\Sigma_{jj1}} = \begin{pmatrix} \sigma^2_{111} & \cdots & \sigma_{1j1} \\ \vdots & \ddots & \vdots \\ \sigma_{j11} & \cdots & \sigma^2_{jj1} \end{pmatrix}$$

The D_{ij} follow the Bernoulli distribution, where π_j is the prevalence of the infection at each lactation stage, ϕ is the multivariate normal probability density function with parameters: $\mathbf{\mu_{j0}}$ ($\mathbf{\mu_{j1}}$) the mean vector and $\mathbf{\Sigma_{jj0}}$ ($\mathbf{\Sigma_{jj1}}$) the variance co-variance matrix for the distribution of the healthy (diseased) animals. Since j = 1,…,4 corresponds to the serum-ELISA measurements and j = 5,…,8 to milk-ELISA measurements on the same individuals, $\pi_j = \pi_{j+4}$ for j = 1,…,4. Given the

distribution of the infected and healthy individuals by lactation stage j, the Se_j and the Sp_j for any cutoff value $c \in (-\infty, +\infty)$ are defined as:

$$Se_j(c) = 1 - \Phi\left(\frac{c - \mu_{j1}}{\sqrt{\sigma^2_{jj2}}}\right)$$

$$Sp_j(c) = \Phi\left(\frac{c - \mu_{j0}}{\sqrt{\sigma^2_{jj0}}}\right)$$

,where Φ is the cumulative distribution function. Subsequently, the ROC curves can be constructed by plotting the pairs of the estimated $\left(1 - Sp_j, Se_j\right)$. The AUC for the serum- and the milk-ELISA at each lactation stage is:

$$AUC_j = \Phi\left(\frac{\mu_{j1} - \mu_{j0}}{\sqrt{\sigma^2_{jj1} + \sigma^2_{jj0}}}\right)$$

for either serum- or milk-ELISA we select as a potential optimum cut-off an S/P percentage that optimizes prevalence-independent summary measures of Se and Sp such as the Youden index $J = \max\left\{Sp_j(c) + Se_j(c) - 1\right\}$. This occurs where the ROC curve gets closest to the top left corner of the graph (Fluss et al., 2005).

Finally, the correlation ρ_{kD} between the serum- and the milk-ELISA for the healthy (D=0) and the diseased (*D*=1) individuals at the kth lactation stage can be estimated by the elements of the variance co-variance matrix: $\rho_{kD} = \sigma^2_{klD} / (\sigma_{kkD}\sigma_{llD})$, with $k = 1, \ldots, 4$ and $l = k + 4$.

***Assuming Constant Prevalence across Lactation Stages*.** Paratuberculosis develops slowly and the prevalence of the disease is expected to remain unchanged across one lactation period. Thus, we also consider a slight modification of our initial model to allow for a constant prevalence across the whole observation period:

$$D_i \sim Bernoulli(\pi)$$

$$(Y_{ij} \left| D_{ij} \right.) \sim \varphi(\cdot \left| \mu_{j1}, \Sigma_{jj1} \right.)^{D_i} \varphi(\cdot \left| \mu_{j0}, \Sigma_{jj0} \right.)^{1-D_i}$$

Prior Selection. We select non-informative priors for the parameters: $\boldsymbol{\pi}$, $\boldsymbol{\pi_k}$, $\boldsymbol{\mu_{jD}}$ **and** $\boldsymbol{\Sigma_{jjD}}$, that follow the Beta (Be), Normal (N) and Wishart distribution respectively:

$$\pi \sim \mathrm{Be}(1,1)$$

$$\pi_k \sim Be(1,1)$$

$$\boldsymbol{\mu}_{\mathbf{jD}} \sim \mathrm{N}(0,100)$$

$$\boldsymbol{\Sigma}_{\mathbf{jjD}} \sim Wishart(8, \boldsymbol{\Gamma})$$

where $\boldsymbol{\Gamma}$ is a $8 \chi 8$ matrix and eight are the degrees of freedom. To represent vague prior knowledge, we chose the degrees of freedom to be as small as possible, eight the rank of $\boldsymbol{\Sigma}_{\mathbf{jjD}}$.

Sensitivity Analysis. We also considered less diffuse prior values, which is recommended when low information priors are used (Ntzoufras, 2009). Two alternative sets of priors were used in the sensitivity analysis. The first set was the same as for the primary analysis but with less diffuse priors specified on the mean of the healthy individuals and on the prevalence of infection: $\boldsymbol{\mu}_{\mathbf{j0}} \sim \mathrm{N}(0,10)$, $\pi \sim Be(2,2)$, $\pi_k \sim Be(2,2)$. The second set was more informative on the same priors: $\boldsymbol{\mu}_{\mathbf{j0}} \sim \mathrm{N}(0.01, 0.07)$, $\pi \sim \mathrm{Be}(15, 2.6)$, $\pi_k \sim Be(15, 2.6)$.

Assesment of Convergence. Convergence diagnostics for MCMC sampling are not foolproof. Therefore, a combination of diagnostics plus visual inspection of the trace plots and summary statistics is recommended (Best et al., 1995). In order to assess the convergence of the Markov Chain Monte Carlo we checked the autocorrelations and the trace plots. We also checked the parameter summary statistics of 90,000 iterations after a burn in phase of 10,000 iterations. This was adequate because the Raftery and Lewis method suggested that analytical summaries of 45,000 iterations after a burn in of 15 iterations were needed. To assess the effect

of prior values selection on the conclusions we obtained the posterior medians and the credible intervals (CrIs) of the AUC's (Choi et al., 2006).

Statistical Software. The model was run in the freeware program WinBugs (Spiegelhalter et al., 1996). The graphs in the manuscript were produced in the statistical package R(http://www.r-project.org/).

2.4 Results

Figure 2.1 shows the distributions of the MAP infected and healthy does for the serum- and milk-ELISA, for each lactation stage. The estimated μ_{j0} and μ_{j1} and the corresponding 95% CrIs for each of these distributions are presented in Table 2.1. Originally, medians of the means and CrIs were obtained for the log-transformed values that were then back transformed to the actual S/P scale. The mean S/P value of both serum- and milk-ELISA did not differ among lactation stages.

The estimated AUCs and CrIs by lactation stage are in Table 2.2. Both tests in all lactation stages were of average (0.7-0.9) overall discriminating ability as measured by the AUC. Both tests had comparable AUCs across the different lactation stages. Further, for either test, there was not a significant difference between the different lactation stages with the exception of the estimated AUC for the milk-ELISA during kidding that had a lowered median value of 63 (95% CrIs 39; 82). For both tests, the highest power to discriminate healthy from infected does was at late lactation.

ROC curves for both tests by lactation stage are in Figure 2.3. Evidently, despite the comparable overall discriminating ability they had different diagnostic accuracy at selected cut-offs. The Se and Sp at the recommended cutoffs (s/p= 45% in serum and 20% in milk) and at the 50% reduced cutoffs (Kostoulas et al., 2006) are in Table 2.1. When the cutoff values were decreased, the Ses were increased without serious loss of Sps. Specifically, the optimum cut-offs that simultaneously maximized Se and Sp of the serum- and milk-ELISA were: 0.45, 0.45, 0.46, 0.47 and 0.44, 0.43, 0.44 and 0.46 at kidding, early, mid and late lactation stage, respectively.

Estimates under the model assuming distinct prevalence for each lactation stage and the one with constant prevalence were comparable (Tables 2.1 and 2.2), indicating that the non-lactation stage-specific prevalence was similar.

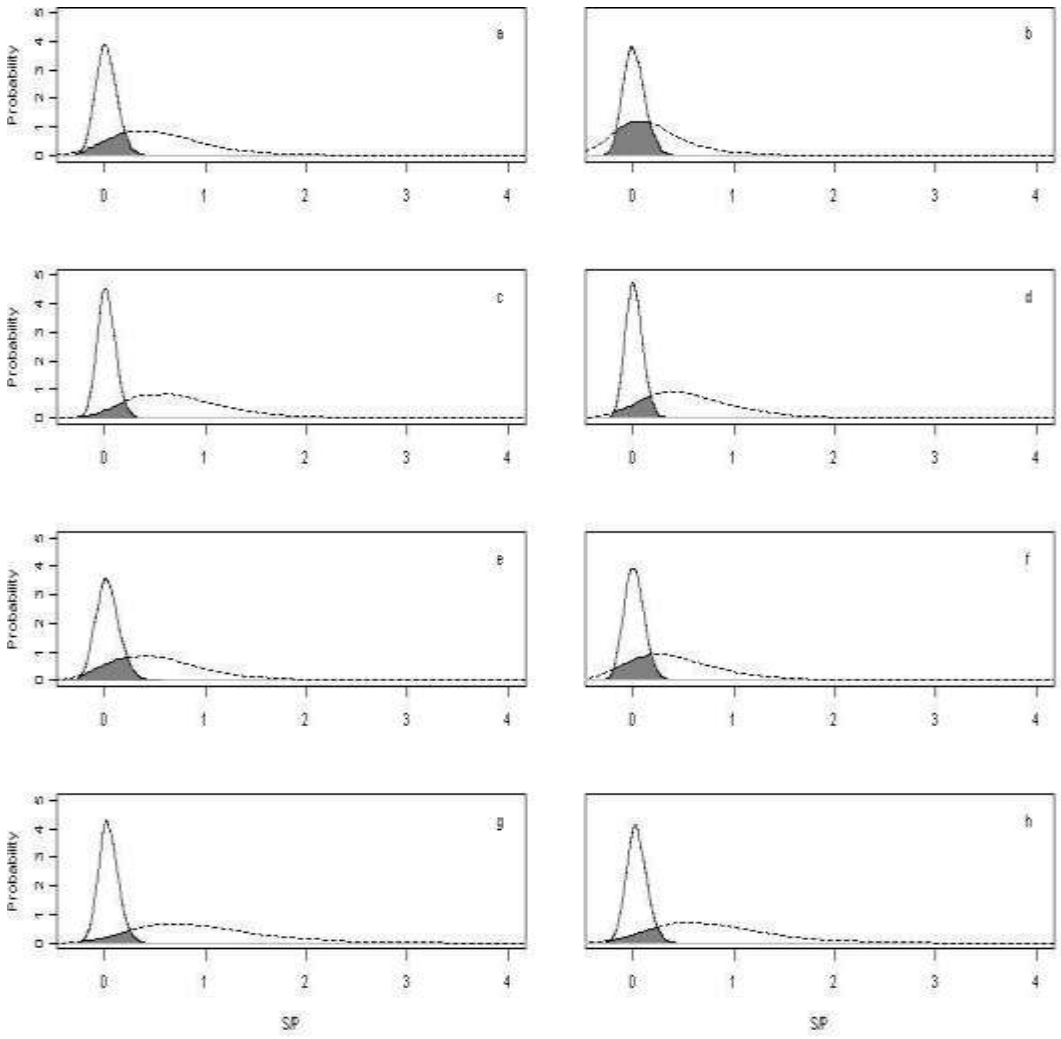

Figure 2.1 . The predicted distributions of the sample to positive ratios (**S/P**) of the healthy and the infected population in serum- (left column) and milk -ELISA (right column) at kidding :(a), (b) , early:(c), (d), mid: (e), (f) and late: (g), (h) stage of lactation. Initial predictions were based on the variable $Y_{ij} = \log_e \{(S/\mathrm{P})+1\}$, which was then back-transformed to the original S/P

percentage. The grey area is the overlap between the healthy and the infected population. Note that the better discrimination ability of the test the smallest the overlap.

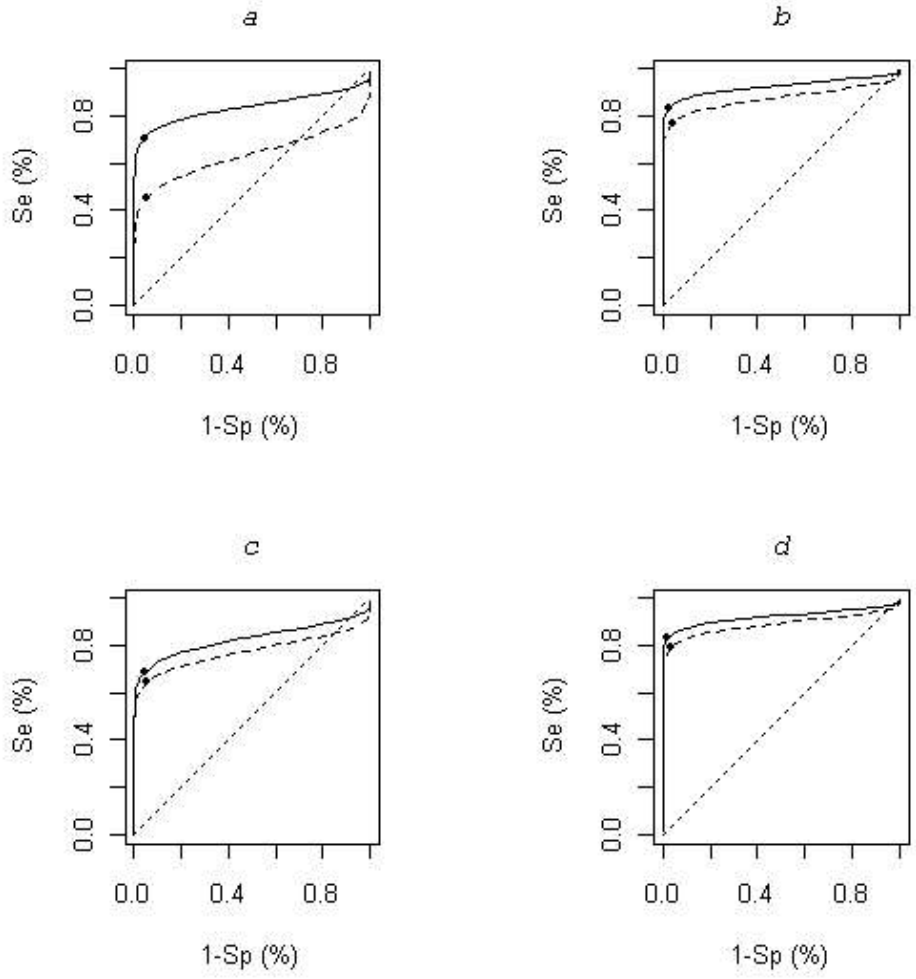

Figure 2.2. Reciever Operating Curves in serum- (solid line) and milk- ELISA (long-dash) at kidding (a), early (b), mid (c) and late (d) stage of lactation. For comparison, the short-dash line depicts the curve of a non-informative test, with area under the curve equal to 0.5.

Table 2.1 The estimated medians of the mean values of the distributions of the healthy and the diseased ELISA responses for each stage of lactation in serum and milk ELISA of the log normal sample to positive ratios (S/P)[1] which was then back transformed.

			Median of the mean S/P ,10^{-2} (95% credible intervals)			
			Log normal		Back Transformed	
Model[2]	ELISA	Lactation stage	Healthy	Diseased	Healthy	Diseased
I	Serum	Kidding	2(0;4)	41(15;67)	37(37;38)	55(43;72)
		Early	2(1;3)	52(32;73)	38(37;38)	62(51;76)
		Mid	3(1;5)	41(15;68)	38(37;39)	56(43;73)
		Late	4(2;5)	63(37;91)	38(38;39)	69(53;91)
	Milk	Kidding	1(-1;3)	14(12;40)	37(36;38)	42(33;55)
		Early	1(0;2)	41(19;66)	37(37;38)	55(44;71)
		Mid	1(-1;3)	33(3;63)	37(37;38)	51(38;69)
		Late	4(2;5)	56(31;83)	38(38;39)	64(50;84)
II	Serum	Kidding	2(0;4)	37(13;62)	37(37;38)	53(42;69)
		Early	2(1;3)	51(31;72)	38(37;38)	61(50;76)
		Mid	3(1;5)	40(14;67)	38(37;39)	55(42;72)
		Late	4(2;5)	58(33;84)	38(38;39)	66(51;85)
	Milk	Kidding	1(-1;3)	12(-10;36)	37(36;38)	42(33;53)
		Early	1(0;2)	40(18;65)	37(37;38)	55(44;70)
		Mid	1(-1;3)	32(3;61)	37(37;38)	50(38;67)
		Late	3(2;5)	55(30;81)	38(38;39)	64(49;83)

[1] $Y_{ij} = \log_e \{(S/P)+1\}$

[2]In model I(II) we assumed different(constant) prevalence for each lactation stage

Table 2.2

Sensitivities and Specificities for the recommended and 50% reduced cuttoffs at different stages of lactation in serum and milk ELISA and the corresponding AUC's

		Median(95% CrIs)					
Model	ELISA	Stage of lactation	Se[a]	Sp[a]	Se[b]	Sp[b]	AUC
I	Serum	Colostrum	50 (28- 73)	100 (100- 100)	67 (43- 86)	96 (92- 98)	81 (60- 94)
		Early	66 (44- 86)	100 (100- 100)	83 (61- 95)	98 (96- 99)	93 (76- 99)
		Mid	53 (30- 77)	100 (99- 100)	70 (45- 89)	94 (90- 97)	82 (60- 95)
		Late	69 (47- 87)	100 (100- 100)	81 (60- 94)	95 (92- 97)	89 (71- 98)
	Milk	Colostrum	43 (21- 68)	95 (91- 97)	53 (29- 76)	79 (72- 84)	63 (39- 82)
		Early	74 (50- 92)	98 (96- 99)	82 (58- 96)	84 (79- 88)	87 (66- 97)
		Mid	63 (37- 85)	95 (91- 98)	71 (44- 90)	79 (72- 85)	77 (51- 93)
		Late	81 (59- 94)	93 (89- 96)	86 (66- 97)	73 (66- 78)	88 (69- 97)
II	Serum	Colostrum	54 (30- 77)	100 (100- 100)	70 (45- 89)	96 (93- 96)	84 (62- 96)
		Early	67 (44- 86)	100 (100- 100)	83 (62- 95)	98 (96- 98)	93 (76- 98)
		Mid	54 (30- 77)	100 (99- 100)	70 (45- 89)	94 (90- 94)	82 (60- 95)
		Late	74 (50- 92)	100 (100- 100)	85 (64- 97)	95 (92- 95)	92 (75- 99)
	Milk	Colostrum	45 (21- 71)	95 (91- 95)	55 (30- 79)	79 (72- 79)	63 (37- 84)
		Early	75 (50- 92)	98 (96- 98)	82 (59- 96)	84 (79- 84)	87 (66- 98)
		Mid	64 (37- 86)	95 (91- 95)	72 (45- 91)	79 (72- 79)	77 (52- 93)
		Late	82 (60- 95)	93 (89- 93)	87 (66- 97)	73 (66- 73)	89 (69- 98)

[a] The recommended cutoff(S/P=40% for serum and S/P=0.25 for milk

[b] The 50% lowered cutoffs

[c] In model II we assume that the prevalence of the disease varies across lactation

2.5 Discussion

We assessed the overall discriminatory power of a serum- and milk-ELISA for the diagnosis of MAP infection in dairy goats at different lactation stages, namely kidding, early- mid- and late-lactation. The estimated AUC, which serves as a global average statistic of the diagnostic validity of a test, indicated that both serum- and ELISA, at each lactation stage, are moderately accurate tests because they fall within the 0.7-0.9 interval (Greiner et al., 2000). The AUC estimates were in accordance with a previous AUC that measured the diagnostic accuracy of a serum ELISA in Greek goats (Kostoulas et al, 2006). In this study they also used latent class models to adjust for all latent infection stages. The latent class models do not lead to overestimates of the diagnostic accuracy of tests, which can occur when the accuracy estimates are based on confirmation procedures that are used as golden standards but do not include all latent cases of infection.

Evidently, the overall discriminatory power of the serum-and milk-ELISA was moderate due to existence of latently infected animals that were at the early infection stages. At the early stages of MAP infection, undetectable levels of antibodies are produced. IFN-γ and tumor necrosis factor-alpha (TFN- α) that activate macrophages and achieve control of the infection usually precede a humoral response in goats (Lyberck et al. 2011; Storset et. al, 2001) and cattle though low level of detectable antibodies could occur at this stage. Clearance may or may not occur because some macrophages remain inactive and infected. These macrophages decay sporadically within granulomas, which account for transient bacterial shedding. As MAP infection progresses the cell-mediated immune reactions are no longer capable of controlling MAP proliferation and a shift to a humoral immune response and production of detectable level of antibodies occurs (Coussens, 2001; Stabel et al., 2000).

The estimated AUCs were comparable between serum- and milk-ELISA across all lactation stages. Therefore, the milk-ELISA may be used instead of the serum-ELISA for the diagnosis of MAP infection in dairy goats. Both tests seem to be of similar discriminatory power

but the former has the advantage that milk samples are easily collected, in a non-invasive way, which offers the farmer the opportunity to screen for MAP with less labor and sampling costs.

The estimated means of the S/P ratio in milk were higher at late lactation than kidding for the infected population (Table 2.2). Nielsen et al (2002b) suggested a similar trend in the milk of dairy cattle. The latter authors also found high antibody levels at the beginning of lactation, which was not observed in this study. Differences in the milking frequency, milk volume and husbandry between dairy cattle and goats could partially explain this. An inverse relationship exists between milk volume and level of IgG concentration in dairy cattle (Pritchett et al., 1991) while the milk IgG concentration is negatively correlated with the milking frequency in goats (Hernández-Castellano et al., 2011). Further, the IgG levels in goat milk depend on the milking frequency and the stage of lactation. In Greek dairy goat flocks, the does are housed indoors with the newborn kids the first five days after kidding. We collected colostrum samples 10h after kidding at a time at which the kid had increased milking frequency. A similar trend was observed in Majorera goats where the colostrum IgG concentration declined rapidly in the first 10 h after kidding (Moreno-Indias et al., 2012). Greek dairy goats are milked once daily at late lactation, leading to a high antibody concentration in milk.

We estimated the optimum cut-offs that simultaneously maximize the Se and Sp of the ELISA, for the sera and milk testing at each lactation stage. The optimum cutoffs were similar with the recommended ones by the manufacturer for the serum-ELISA. However, the recommended cut-off for the milk-ELISA was lower than the optimum one. The manufacturer proposes one cut-off for the blood- and the serum-ELISA, which is not species specific. However, differences may exist in the distribution of MAP strains, immune response, ability to contain infection and clinical manifestations between cattle, sheep and goats. Thus, a species specific approach is preferable (Kostoulas et al., 2006). Variable stains of MAP stimulate variable levels of antibody response in goats and cattle. The major strain types, S- and C-type, are not host specific (de Juan et., al 2005; Sevilla et al., 2007). In a recent study in Northern Greece the authors found significant genetic diversity of MAP isolates in small ruminants (Dimareli-Malli et al., 2013). In terms of clinical disease, goats appeared more susceptible to MAP infection, whether infected with S- or C– type, than sheep with cattle being the most resistant (Stewart et al., 2007). Other papers that applied the same ELISA, at the recommended cut-offs, in dairy cattle and goats found an agreement between the proportion positive in milk-ELISA with that in

fecal culture, while the proportions positive in serum-ELISA and fecal culture disagreed (Salgado et. al, 2007; Hendrick et al., 2005).

When we lowered the recommended cut-off we improved the Se without serious loss of Sp (Table 2.1), which is in accordance with previous findings in goats (Kostoulas et al., 2006). The suggested optimum cut-offs that simultaneously maximize the Se and Sp correspond to an informed decision that assigns equal weights to the cost of the false positive and the false negative test outcomes and implies that the prevalence in the target population is about 50%. This approach may not always fully exploit the information provided by the diagnostic test in the context of a particular diagnostic objective, but facilitates comparison of different diagnostic tests. Cut-off selection is an informed procedure that takes into account the epidemiological situation in the target population and the relative consequences of false negative and false positive test results that are defined on the grounds of a specific decision making situation (Greiner et al., 2000) and may not necessarily be set to equal. Kostoulas et al. (2006), provided different cut-offs for the serum-ELISA in sheep and goats for variable prevalence schemes and ratios of relative costs. However, our approach gives parity-specific distributions of the healthy and the infected dairy goats that permit the continuous interpretation of test results. The continuous interpretation eliminates the loss of information that occurs under dichotomization of continuous test results. Dichotomization of continuous results leads in loss of valuable information because the information conveyed in the test result is reduced to considering all positive results equal. Hence, potential associations between the continuous test result and risk factors or productivity indices is attenuated or lost. A diagnostic interpretation approach that utilizes the actual continuous responses has been recently proposed (Toft et al., 2005) and utilized in the identification of the different stages of MAP infection. Decision making, such as culling or no culling, can be based on this continuous interpretation in connection with productive and reproductive indices of dairy cattle (Nielsen et al., 2007; Toft et al., 2005).

The sensitivity analysis suggested that the posterior distributions were robust under alternative prior information. Specification of more informative priors gave similar results with the primary analyses that included vague prior information. We also considered two different variations of the same model, one assuming a different prevalence of MAP infection for each lactation stage (primary analysis) and one setting MAP prevalence constant across all lactation stages. Prevalence, AUC, Se and Sp estimates under the alternative model specifications were

similar (Table 2.1) indicating that due to the low progression of MAP infection the prevalence of MAP can be considered relatively constant during the 14 month follow-up period. In endemic situations MAP infection can be expected to develop slowly over time (Chiodini et al., 1984).

The milk-ELISA can be as accurate as serum-ELISA across all lactation stages and especially at late lactation. Also, there is no need for lactation-stage specific selection to detect the disease as the prevalence is constant. Milk-ELISA could be preferred to the serum-ELISA as milk sampling is a non-invasive, rapid, easy to apply and low cost procedure and could serve as the diagnostic tool of choice during the implementation of MAP control programs that require frequent testing. Under such programs, interpretation of the actual continuous milk-ELISA results in connection with productive indices can be used to enhance control options.

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Chapter 3

Bayesian validation of a commercial serum/milk ELISA across lactation in Greek dairy sheep

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3.1. Abstract

A total of 854 paired milk and blood samples were collected from ewes of a Greek flock and used to validate a commercial (IDEXX Pourquier, Montpellier, France) serum/milk ELISA against Mycobacterium avium subsp. paratuberculosis across lactation. We implemented Bayesian mixture models to derive the distribution of the responses of healthy and infected ewes. Both serum and milk ELISA had low to average overall discriminatory ability as measured by the area under the curves and comparable sensitivities and specificities at the recommended cut-offs. Lowering the cutoffs led to an increase in the sensitivities without serious loss in specificities.

3.2. Introduction

Paratuberculosis is a chronic intestinal disease, caused by *Mycobacterium avium subsp. paratuberculosis* (MAP), with worldwide distribution in domestic and wild ruminants and significant economic impact (Harris and Barletta, 2001). In dairy cows, repeated testing of serum or milk by ELISA to detect the humoral immune response against MAP at specific lactation stages was shown to improve the diagnostic sensitivity (Se) (Nielsen et al., 2011). Compared to microbiological fecal testing especially the milk ELISA has the advantage of low cost sample collection, because farmers repeatedly collect and submit milk samples to be tested for other reasons, may be automated and provide results faster and at a fraction of the cost. It is, therefore, attractive for monitoring large non-vaccinated populations (Oprin et al., 2012) of dairy sheep and goats, as is the Greek national flock, reared across the country's territory. We recently estimated the Se and Sp across lactation of a commercial indirect serum/milk ELISA in dairy goats (Angelidou et al., 2014). For dairy sheep, estimates of its diagnostic validity across lactation are lacking. Those reported by researchers (Munjal et al., 2004) for another milk ELISA were based on cases of paratuberculosis confirmed by pathological changes. However, MAP infection often may be missed by histopathological examination, either because the pathologists were imperfect or because the bacteria had not yet caused detectable pathological changes (Whittington et al., 1999). Hence, such evaluations did not include all latent cases of infection Nielsen et al., 2002) and the published estimates are in reality relative Se and Sp estimates to an imperfect diagnostic test. We estimated the diagnostic validity of a serum ELISA in Greek dairy sheep by non-gold standard methods (Kostoulas et al., 2006) . We did not, however, assume repeated testing across lactation. Thus, in this study, we estimated the diagnostic validity of a commercial indirect serum/milk ELISA across lactation in Greek dairy sheep. We implemented a Bayesian mixture model that adjusted for the potential correlations between repeated measurements at different lactation stages.

3.3. Materials and methods

3.3.1 Study Population and Sampling Scheme

We collected paired sera and colostrum/milk samples from 108 randomly selected ewes from the 400 ewes of a Greek dairy sheep flock with history of clinical paratuberculosis, which was unvaccinated against MAP. The ewes belonged either to the domestic breeds Chiotiko, Karagouniko and Frizarta or to their crosses. The animals were kept under semi-intensive management for milk production, which was the primary breeding goal. They grazed on pasture most of the year and were additionally fed concentrates in the shed when in milk. They were mated to rams, in an unsupervised manner, in July-August and delivered during January-February of the following year. The lambs were weaned 25–30 days after birth; subsequently the ewes were hand milked 2-3 times daily. Milking was ceased abruptly after approximately eight months when the farmer felt that the milk yield was so reduced that it did not pay off the milking routine and the extra feeding. The selected ewes were followed up from January to August 2014. We collected a total of 854 blood and milk/colostrum samples during the eight-month-long lactation period. Each ewe was sampled at four consecutive times starting from lambing and covering the early, mid and late lactation.

3.3.2 Diagnostic Tests

The sera and milk/colostrum samples were tested for antibodies against MAP with a commercially available indirect ELISA (IDEXX Paratuberculosis Screening Ab Test) following a previously described procedure (Angelidou et al., 2014). The recorded optical densities were transformed to the sample-to-positive (S/P) ratio, which was kept on a continuous scale.

3.3.3 Statistical Analyses

We implemented a Bayesian mixture model in order to predict the distribution of the serum and milk/colostrum ELISA response, among MAP-infected and uninfected ewes, separately for each lactation stage. A thorough description of the model can be found in Angelidou et al., 2014. Briefly, the model determines the distribution of the continuous ELISA responses by infection status and lactation stage, while adjusting for the likely within animal and

lactation stage correlation. Given the distribution of ELISA responses, the area under the estimated curves (AUC) for the serum and milk ELISA at each lactation stage was calculated. Then, the Se and Sp for any cutoff value were estimated. Subsequently, the Receiver Operator Characteristic (ROC) curves were constructed by plotting the pairs of the estimated 1 − Sp, Se. For either serum or milk ELISA, we selected as optimum cutoff the S/P percentage which optimized the Youden index (Fluss et al., 2005). Priors specific to the mixture model analysis were as follows: we specified the mean for the infected individuals to be $\boldsymbol{\mu}_{j1} \sim \text{beta}(2,5)$ and for the non-infected to be $\boldsymbol{\mu}_{j1} \sim \text{beta}(1.5,54.3)$.

3.4 Results

Figure 3.1 shows the distributions of MAP infected and non-infected ewes for the serum and milk ELISA, for each lactation stage. ROC curves for both tests by lactation stage are in Figure 3.2. The median values of the estimated Ses at the manufacturer recommended cutoffs (S/P = 45% in serum and 20% in milk) were: 43 [95% Credible Intervals (CrIs):22;62], 42 (22;61), 45 (28; 69) and 44 (26;67) for the serum ELISA , and 50 (35;72), 51 (36;74), 56 (41;85) and 56 (40;84) for the milk ELISA during lambing, early, mid and late lactation, respectively. Sps, at the same cutoffs, for the serum ELISA were: 97 (92;99), 98 (95;100), 96 (91;99) and 98(94;99) for the milk ELISA: 83(74;90), 87(79;93), 80(72;88) and 78(69;86) for each lactation stage, respectively. When the cutoff values were reduced, Se was increased without serious loss in Sp (Table 3.1). The optimum cutoffs that simultaneously maximized the Se and Sp were: 0.31, 0.29, 0.31, 0.29 for serum and 0.34, 0.29, 0.34, 0.34 for milk for each lactation stage, respectively. Finally the corresponding medians of the AUC for the serum and milk ELISA by lactation stage were: 61% (50;84), 61% (51;84), 65% (51;91), 65% (51;89), 60% (50;82), 61% (50;84), 67% (51;91) and 66% (50;90). Both tests in all lactation stages were of low to moderate overall discriminating ability and had comparable AUCs across the different lactation stages which did not differ between the different lactation stages.

Table 3.1 The medians (95% credible interval in parenthentheses) of the area under the curve (AUC), and sensitivity (Se) and Specificity(Sp) for the recommended and 50% reduced cuttoffs at different stages of lactaction in serum and milk ELISA .

	Stage Of lactation	Se[a]	Sp[a]	Se[b]	Sp[b]	AUC
Serum	Lambing	43(22;62)	97(92;99)	49(34;72)	88(80;94)	61(50;84)
	Early	42(22;61)	98(95;100)	49(33;71)	91(84;96)	61(51;84)
	Mid	45(2869)	96(91;99)	53(38;80)	86(79;93)	65(51;91)
	Late	44(26;67)	98(94;99)	52(37;78)	89(81;94)	65(51;89)
Milk	Lambing	50(35;72)	83(74;90)	55(43;79)	69(60;76)	60(50;82)
	Early	51(36;74)	87(79;93)	55(44;80)	73(64;80)	61(50;84)
	Mid	56(41;85)	80(72;88)	62(46;91)	67(58;75)	67(51;91)
	Late	56(40;84)	78(69;86)	62(46;89)	65(54;72)	66(50;90)

[a]The recommended cutoff(S/P=40% for serum and S/P=0.25 for milk)

[b] The 50% lowered cutoffs

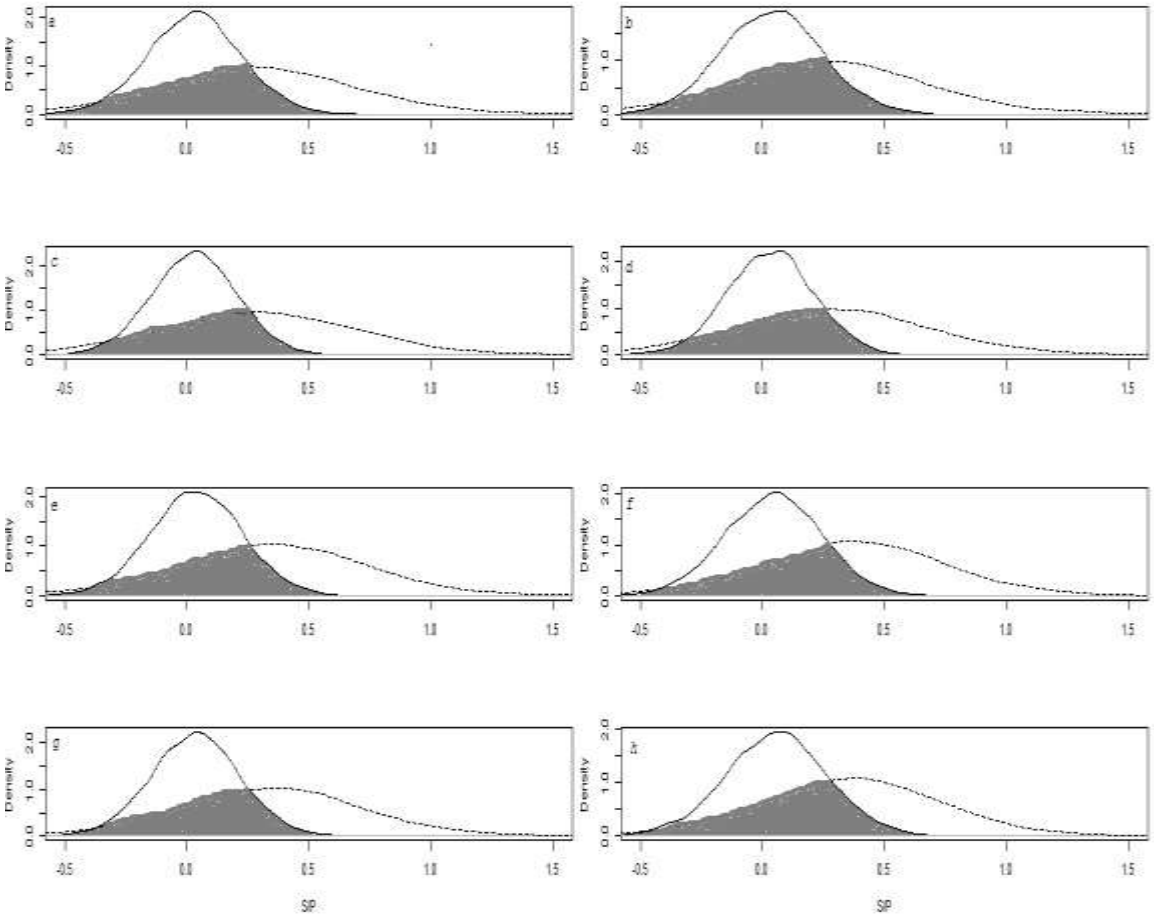

Figure 3.1.The predicted distributions of the sample-to-positive ratios (S/P) of the healthy and the infected population in serum (a, c, e, g) and milk (b, d, f, h) ELISA at (a, b) lambing, (c, d) early, (e, f) mid, and (g, h) late stage of lactation. Initial predictions were based on the variable Yij = loge [(S/P) + 0.8]+0.25, which was then back-transformed to the original S/P percentage.

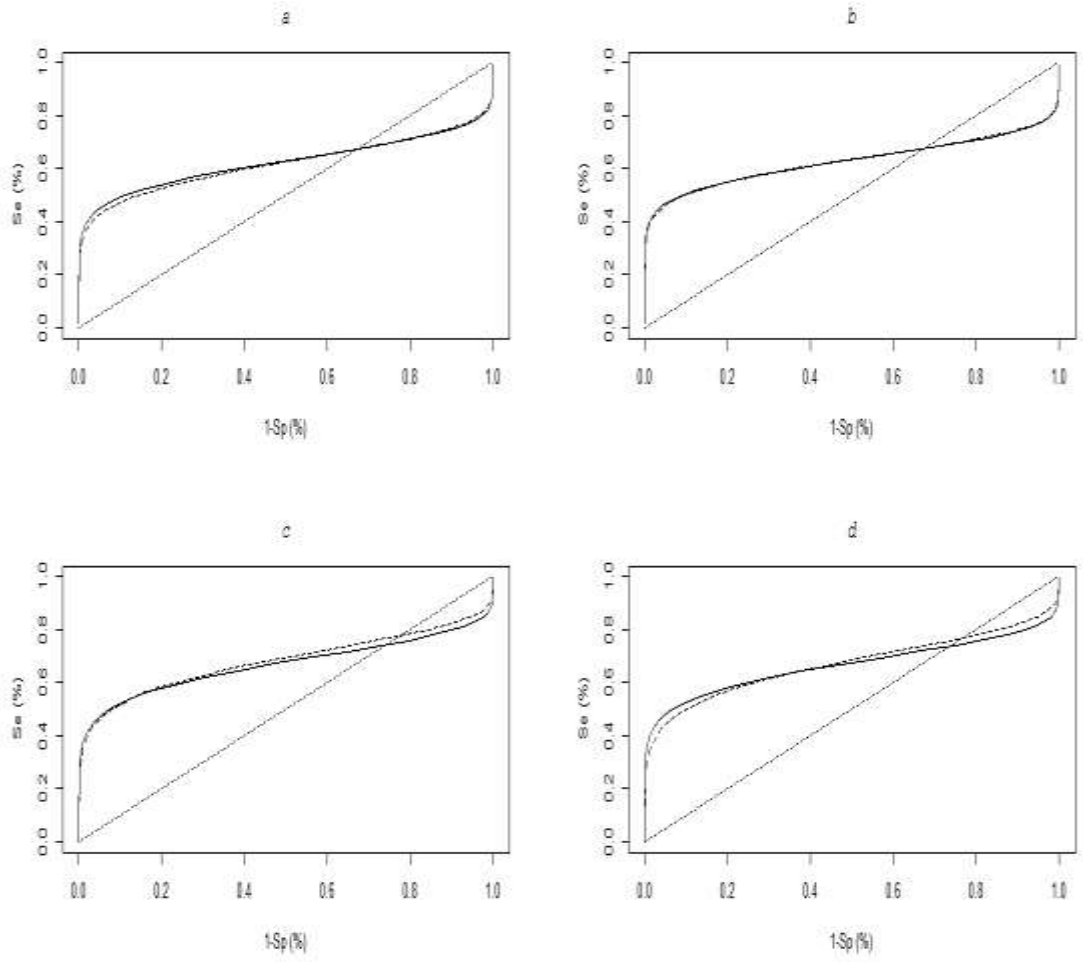

Figure 3.2. Receiver operating curves in serum (solid line) and milk (long-dash) ELISA at (a) kidding, (b) early, (c) mid, and (d) late stage of lactation. For comparison, the short-dash line depicts the curve of a noninformative test, with the area under the curve equal to 0.5.

3.5 Discussion

We assessed the overall discriminatory power of a serum/milk ELISA for the diagnosis of MAP infection in dairy sheep at different lactation stages, namely at lambing, in early, mid and late lactation. The estimation method applied provided lactation-specific distributions of MAP-infected and non-infected dairy sheep which permitted continuous interpretation of test results.

In dairy cows, researchers (Toft et al.,2005) proposed that the actual continuous responses can be utilized in the identification of the different MAP infection stages. In dairy sheep the serum and milk ELISA had comparable AUCs which did not differ by lactation stage. Their diagnostic accuracies were low to moderate (greiner and Gardner, 2000) mainly because of the low Ses. Therefore, despite its low cost of sampling and testing, the serum/milk ELISA may not be the preferred diagnostic method for monitoring unvaccinated sheep populations. In contrast to sheep, in dairy goats the same ELISA had higher diagnostic accuracy (Angelidou et al., 2014). Further, the cutoffs that simultaneously maximized the Se and Sp of the ELISA, for either serum or milk testing were lower than those recommended by the manufacturer and those we proposed for goats (Angelidou et al, 2014). These findings highlight the need for species specific cutoff selection since there are differences in the distribution of MAP strains, immune response, ability to contain infection and clinical manifestations between sheep and goats(Stewart et al., 2006).

3.6 References

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Chapter 4

Flock-level factors associated with the risk of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection in Greek dairy goat flocks.

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4.1 Abstract

In this cross-sectional study we identified flock-level risk factors for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection, in Greek dairy goat flocks. We collected 1599 milk samples from does that were at the last stage of lactation in 58 randomly selected dairy goat flocks, during May to September 2012. The collected samples were tested with a commercial milk ELISA (IdexxPourquier, Montpellier, France) and the results were interpreted at a cut-off that optimized the accuracy of the diagnostic process. For the analysis of the data we used Bayesian models that adjusted for the imperfect Se and Sp of the milk-ELISA. Flock was included as a random effect. Does in flocks that used common water troughs and communal grazing grounds had 4.6 [95% Credible Interval (CI):1.5; 17.4] times higher odds of being MAP-infected compared to does in flocks that had no contact with other flocks. Does of flocks supplied with surface water from either streams or shallow wells had 3.7 (1.4; 10.4) times higher odds of being infected compared to those in flocks watered by underground and piped water sources.When kids were spending equal to or more than 10 hours per day with their dams they had 2.6 (1.1; 6.4) times higher odds of being MAP infected compared to those where kids that were separated from their dams for less than 10 hours per day. Finally, does in flocks that continuously used the same anti-parasitic compound had 2.2 (1.0; 4.6) times higher odds of MAP infection compared to those in flocks alternating anti-parasitic compounds. These results should be considered in the development of a nationwide future control program for caprine paratuberculosis in Greece.

Key words: Paratuberculosis; Goat flock; Milk ELISA; Risk factor

4.2 Introduction

Paratuberculosis (Johne's disease) is a chronic intestinal infection of global importance in mainly domestic and wild ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). MAP infection of small ruminants has worldwide distribution, recognized in sheep and goats in many countries, including the southern hemisphere in Australia, New Zealand and South Africa, numerous northern hemisphere countries, particularly Great Britain, Norway and Austria, with increasing recognition in Mediterranean countries including Greece, Spain, Portugal, Morocco and Jordan (Benazzi et al., 2010;Djønne, 2010; Hailat et al., 2010). Caprine paratuberculosis is also recognised in Turkey, France, Norway, Switzerland, Croatia, Canada, the USA and Chile (Barkema et al., 2010). MAP infection mostly results from fecal-oral route exposure. Fecal-oral route exposure may occur from: (1) ingestion of fecal material from an infected animal, particularly on the teat of an infected dam, plus exposure to manure contaminated pasture, water, supplements or hay contaminated with fecal material from an infected adult animal (Windsor and Whittington, 2010) and (2) the drinking of contaminated colostrum or milk as MAP is also excreted in the colostrum and milk of sheep and goats (Lambeth et al., 2004; Nebbia et al., 2006).Pre-natal infection is also now well described (Lambeth et al., 2004; Whittington and Windsor, 2009). The clinical manifestations of paratuberculosis in goats include progressive wasting and decrease in milk production, which are followed by the manifestation of advanced clinical disease: flafy skin, poor hair coat, progressive emaciation, dehydration, anemia with submandibular edema, depression, and diarrhea (Stehman, 1996). Paratuberculosis was first recognized in Greek goats in 1975 (Leontides et al. 1975). Today, the majority of Greek goat flocks are endemically infected with MAP (Ikonomopoulos et al., 2007;Dimareli-Malli et al., 2013). Greece has the largest goat herd in the EU accounting for around 50% of the EU total and is self-sufficient in goat-meat (http://lhu.emu.ee/downloads/Welfood/WP1T2L4.pdf). The Greek national herd comprises of approximately 4 million goats, which are reared primarily for milk production (Food And Agriculture Organization of The United Nations Statistics Division). The main reason why there are so many goats in Greece is because there is a strong tradition of cheese consumption in the

Greek gastronomy; cheese is not a food supplement, it is food. Contrary to its European counterparts of France, Italy and Spain, Greeks consume cheese at all times, i.e. for breakfast, lunch, dinner, alone or with other food, having the highest consumption in EU of 23 kg per person per year. A plethora of protected destination of origin (e.g. feta) or protected geographical indication cheeses of Greece are dependent on the production of goat milk. In a study on the prevalence of MAP in retail feta cheese (produced from sheep and goat milk) the authors reported 50% (21/42) and 4.7% (2/42) PCR- and culture-positivity, respectively, for MAP (Ikonomopoulos et al., 2005).A potential zoonotic link between MAP and human inflammatory bowel diseases including Crohn's disease has been suggested but remains unclear (Over et al., 2011). If MAP is confirmed as a zoonotic pathogen, public confidence in products of the dairy industries is very likely to decline.

Within an infected flock most animals acquire MAP early in their life. Susceptibility to infection decreases over time, while environmental(Tiwari et al., 2009) and genetic (Koets et al., 2000) factors, which have not been fully conceptualized yet, playing a critical role on whether initial entrance and persistence of MAP will lead to clinical manifestations, be restrained during the productive life of infected animals or even be cleared out (Kostoulas et al., 2010). Although they are important for the development of national control programs, few studies aiming to identify risk factors for caprine paratuberculosis have been carried out. Ideally, the programs should depend on a risk-based system with a framework for identification of high risk, for the spread of MAP infection, flocks and regions. A Spanish study reported that factors related to intensive management such as herd size, foreign breeds and high replacement rate were associated with MAP infection (Mainar-Jaime and Vazquez-Boland, 1998). Addition of new animals and mixed farming were also found as factors associated with increased risk of paratuberculosis in goats (Al-Majali et al., 2008). However, in a recent study no associations were detected (Martinez-Herera et al., 2012). Unfortunately, these studies ignored the fact that diagnostics for MAP are imperfect. Their estimates were not adjusted for the Sp and, most importantly, the low to average Se of MAP diagnostics. In the absence of perfect diagnostic tests and when the misclassification is non-differential odds ratio estimates are usually biased towards the null unless the analysis corrects for test accuracy (Copeland et al., 1977). Methods exist for obtaining corrected odds ratios by incorporating prior information from external estimates on the tests' Se and Sp (McInturff et al., 2004).

We conducted this cross sectional study in order to identify factors associated with the risk of MAP infection in Greek dairy goat flocks. Sampling was conducted during a period for which we demonstrated that the overlap between the distributions of the ELISA responses -the sample to positive ratio- in milk of the healthy and the MAP-infected does is the smallest (Angelidou et al., 2014). In the analysis, we employed Bayesian models to account for the imperfect Se and Sp of the diagnostic test.

4.3 Materials and Methods

4.3.1 Target population and sampling scheme

Goat farming in Greece is a sector of animal production that is generally friendly to the environment usually taking place in disadvantaged for agriculture, hilly and mountainous areas. The animals are kept under semi-intensive management for milk production. The farmers select replacements among the daughters of high-yielding does. The males bought into the flocks originate from high-yielding animals from other flocks. The animals graze on communal pastures throughout most of the year and are additionally fed concentrates. They spend most of the day outside and are moved into the shed during the night. They are mated to bucks, in an unsupervised manner, in June–August and deliver from November to January of the following year. The kids are weaned 15–30 days after birth; subsequently the dams are mechanically or manually milked, twice daily. The milking duration is approximately 5 months; it is ceased gradually or abruptly when the farmer decides that the yield is low to justify the milking routine. The annual replacement risk is approximately 25%, which is the same as the culling risk because the farmers receive European Union-subsidies on the basis of flock size.

The target population included flocks in the region of Thessaly, at the center of the Greek mainland, which were managed semi-intensively for milk production. The animals belonged either to indigenous breeds (i.e. Vlahiki, Eghoria, Paggaio, Skopelos)or crosses of the indigenous with foreign breeds(i.e. Alpine, Zaanen, Damascus, Maltese).All the does of the flocks were unvaccinated against MAP. The sample size employed in this study was selected to detect an expected difference of 6% in the prevalence between the exposed group (11%) and non-

exposed group (5%) to communal grazing/watering with other flocks (based on unpublished data). The sample size was estimated assuming a 95% confidence interval (type I error = 5%) and 80% power (type II error = 20%) and an intra-class correlation coefficient 0.05, adding 20% to the minimum required sample size (of 1200 does, obtained by sampling 48 flocks with 25 does in each flock) to account for the loss of power associated with controlling for confounders (Hintze, 2014).

From 58 flocks we sampled milk from 1599 does from May to September 2012. The sampled flocks were selected with simple random sampling (with the aid of computer-generated random numbers) from the sampling frame of flock identification numbers in the region. Within the flocks the does were selected with systematic random sample while the animals entered the milking parlor.

The mode within flock sample size was 48 does but ranged between 20 to 50 does depending on the size of the flock and the number of non-dry animals at the sampling day. All samples were collected during the late stage of lactation because we recently demonstrated that although in Greek dairy goats both serum and milk ELISA, in all lactation stages, have similar overall discriminatory ability, the smallest overlap between the distributions of the ELISA responses -the sample to positive ratio- in milk of the healthy and MAP-infected does was detected in late lactation (Angelidou et al., 2014).

4.3.2 Diagnostic tests

The milk samples were centrifuged (1200 X g for 20 min), skimmed and stored at -21^{o}C until testing with a commercial indirect ELISA kit (IDEXX® Pourquier, Montpellier, France)using the manufacturer's protocol for bovine milk (Salgado et al., 2005). The recorded optical densities (OD) were transformed to the sample-to-positive (S/P) ratio and were interpreted at the cut-off of 0.35 (Angelidou et al., 2014).

4.3.3 Questionnaire

We developed a questionnaire, in order to collect data on factors that could be associated with the risk of MAP infection in goats. Questionnaire development was based on previously published work in sheep (Lugton et al., 2004) – due to the absence of relevant reports in dairy goats – and expert opinion. Questionnaire data included information on flock size, housing conditions, breed type, production parameters, managerial strategies, manure management, biosecurity measures, disease prevention and nutrition (Appendix 3).

Seventy two questions were included on flock-level factors. Twenty six were closed (e.g. yes/no, always/frequently/seldom/never or pre-set options), thirty were semi-closed (e.g. information on number of days, application frequencies of certain procedures) and the remaining were open-ended (e.g. product names, descriptions) questions. The questionnaire (Appendix 3) was administered and filled through a face to face interview of the farmers by the first author who had no prior knowledge of the MAP infection status of the flocks. Whenever possible, the interviewer checked the accuracy of the information provided by the owner, such as shelter ventilation, by inspecting the facilities.

4.3.4 Statistical Analyses

Definition of infection status. Bayesian mixture models create their own probabilistic definition of infection, which implicitly assumes a biological definition that has to be explicitly described. Essentially, this is determined by the target condition that the analytes and biomarkers of the test under consideration measure (Gardner et al., 2011). In our case, to describe MAP infection in biological terms, we mean that goats carry MAP intracellularly; substantial replication need not take place because the infection can be latent. Entrance and persistence of MAP have lasted long enough to give a detectable humoral immune response at any time during their life; we assumed that once an animal has an established infection, the infection persists for life (Angelidou et al. 2014; Kostoulas et al. 2006; Nielsen et al. 2002).

Bayesian model specification. We employed a Bayesian logistic regression model that adjusted for imperfect Se and Sp of the diagnostic test. Let the variable r_i indicate the number of positive does out of the n tested does with milk ELISA of the ith flock. We assume that r_i is distributed binomially,

$$r_i \sim Binomial\ (Ap_i,\ n_i) \qquad (1)$$

, where Ap_i is the apparent seroprevalence of the ith flock. Let $T+$ denote that a milk sample of a doe has tested positive and let $D+$ denote that the doe has the target condition. We define Se and Sp of the milk ELISA to be, $Se = Pr\ (T+/D+)$, and $Sp = Pr(T-/D-)$, respectively. We also let Tp_i denote the true prevalence of MAP infection in the ith flock. Adjusting for the Se and the Sp of the milk ELISA the apparent seroprevalence of the ith flock is

$$Ap_i = Se \times Tp_i + (1 - Sp) \times (1 - Tp_i) \qquad (2)$$

Then, we model the Tp_i as the logit function of the vector, X_{ij}^T ,where j is the number of the predictor variables including the intercept in the model:

$$Logit(Tp_i) = X_{ji}{}^T \beta_j + u_i \qquad (3)$$

The term $X_{ij}^T \beta_j$ is referred to as the linear predictor (McCullagh and Nelder, 1989)and u_i is indicating the flock random effect. Further, we consider the normally distributed random effect level u_i, with zero mean and a random effects variance σ^2_u.

$$u_i \sim N(0,\ \sigma^2{}_u) \qquad (4)$$

The standard method for specifying priors on β's is to use a multivariate normal distribution (Spiegelhalter et al., 2003). We preferred to obtain conditional mean priors (CMPs) as described by Bedrick et al. (1996). CMPs are constructed from the success probability of different covariate patterns. Briefly, instead of eliciting independent prior information about β's directly we specify uncertainty about probabilities of the disease/infection state being present for various covariate patterns. For *j* regression coefficients (including the intercept), we specify prior information about *j* probabilities of success (disease/infection state being present)

for *j* distinct covariate patterns. Subsequently, the priors on b were induced from the inverse covariance matrix (see Appendix A for a WinBUGS implementation).

Finally we use the Markov chain Monte Carlo samples from the posterior distribution of the β's to make inferences for the odds ratios. Thus we calculate the odds ratio as the exponential function of the regression coeficients (see Appendix for a WinBUGS implementation).

Prior specification. We subsequently specified CMPs about the probability of an animal being sub-clinically infected for each level of the predictor and the intercept. We incorporated prior information about the prevalence of five combinations of covariate patterns, based on the expert opinion of the authors PK and LL, because there were five predictors in the final model (including the intercept). The specified covariate patterns with the corresponding input probabilities are in Table 4.1. In the absence of available information, non-informative, uniform beta distributions can be defined for the probabilities of success of the distinct covariate patterns.

The prior information about the Se and the Sp of the test is incorporated in the model in the form of beta distributions (Table 4.1):

$$Se \sim \text{beta}(\alpha_{Se},\beta_{Se}) \text{ , Sp}\sim\text{beta}(\alpha_{Sp},\beta_{Sp}) \qquad (5)$$

Finally, we specify a non-informative prior on the inverse of the random effect variance:

$$1/\sigma_u^2 \sim \text{gamma}(0.001, 0.001) \qquad (6)$$

Model Building. For model building, seventy eight candidate variables were initially examined. When pairs of highly correlated variables were encountered, selection of the variable to be included in the model was based on biological plausibility. Twenty five variables were dropped due to high correlations. The remaining twenty variables were screened, one-by-one, using a univariable approach (Martin, 1997) in the Bayesian logistic regression model specified in Section 2.4.2. We incorporated non-informative, uniform beta distributions for the probability of success of the distinct covariate patterns. During this screening phase, a significance level of P≤ 0.25 was used (Mickey and Greenland, 1987). We approximated the

classical *P*-values in the Bayesian framework using the posterior densities of the beta distributions.

All twenty variables found significant, were simultaneously offered to a full model which was, subsequently, reduced by backwards elimination (Hosmer and Lemeshow, 1989), until only those significant at $P \leq 0.05$ remained. Finally, a stepwise forward selection process was done by offering previously excluded variables to the final model one at a time. During the model building, we incorporated non-informative, uniform beta distributions for the probability of success of the distinct covariate patterns.

Assessment of convergence. To assess the convergence of the Markov Chain Monte Carlo (MCMC), we checked the autocorrelations and the trace plots. We also checked the parameter summary statistics of 50,000 iterations after a burn-in phase of 50,000 iterations.

Statistical software. All models were built and run in the freeware program WinBUGS (Spiegelhalter et al., 2003). WinBUGS code with detailed step-by-step explanations and the CMPs specification can be found in the Appendix. WinBUGS was also used for checking the autocorrelation plots. To calculate the parameters of the beta prior distributions we utilized the Betabuster software, which is public domain software available at http://www.epi.ucdavis.edu/diagnostictests.

4.4 Results

Flock sized ranged from 45 to 650 does (median 160). In 14/58 (24.1%) flocks there was at least one test-positive doe. In these test-positive flocks the mean within-herd prevalence was 10% (0.08; 0.12).

After uni-variable screening and pairwise correlation analysis the variables with $P \leq 0.25$ further considered in multivariable analysis included the information from the administrated questionnaire (Appendix 3): 1) Housing conditions; flooring, altitude, kind of roof, 2) water supplied to the flock; origin of the water from surface, 3) exposure of the kids post partum; where the does of the flock usually deliver, applied disinfectant to the matertinity paddock, 4) exposure of the kids during suckling; kids' spending hours per day with their does, food and water sharing of the kids with the does , 5) production parameters; culling rate per year, age

category at culling, 6) biosecurity; replacing rate, communal grazing with other flocks, contact with wildlife during grazing, 7) gastrointestinal parasite control; compound combination, alternating use of antiparasitic compounds, 8) nutrition; type of additional bulk food providing in the shed, additional supplements containing minerals providing to the does and 9) Soil PH-Manure management; disinfection applied per year with limestone, frequency of cleaning, disposal location of the manure.

The final model included four factors: the origin of the water from surfaces, the contact with other flocks, the kids' spending equal to or more than 10 hours per day with the dams and the alternating use of different anti-parasitic compounds. The frequency distributions of the significant variables offered to the final Bayesian logistic regression model are in Table 4.2. The odds ratios estimated under the Bayesian model that accounted for the imperfect Se and Sp of the milk ELISA are in Table 4.3. Specifically, does of flocks which used common water troughs and communal grazing grounds had 4.6 [95% Credible Interval (CI):1.5; 17.4] times higher odds of being MAP-infected compared to does of flocks that had no contact with other flocks. Does of flocks supplied with surface water from either streams or shallow wells had 3.7 (1.4; 10.4) times higher odds of being infected compared to those in flocks which were watered by underground and piped water sources. Does in flocks where the kids were spending equal to or more than 10 hours per day with their dams had 2.6 (1.1; 6.4) times higher odds of MAP infection than those in flocks where the kids were separated from their dams for less than 10 hours per day. Finally, does in herds that continuously used the same anti-parasitic compound had 2.2 (1.0; 4.6) times higher odds of MAP infection compared to those in flocks commonly alternating anti-parasitic compounds (the inverse association is in Table 4.3). Finally, the flock level variance was 0.8 (0.1; 2.0).

Table 4.1. Priors for the sensitivity(Se) and specificity (Sp) of the milk ELISA at the selected cutoff (0.35) and conditional mean priors (CMPs) on the expected risk of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection for specific combinations of the fitted covariates (covariate

patterns) in the final model.

Covariate pattern					Prior Specification		Mode
Intercept	Surface Water	Contact with other flocks	Kids' spending ≥10 hours per day	Alternating use of antiparasitic compounds	Se	Be(20.3, 10.08)	0.68
					Sp	Be(315.32,1.6)	0.99
1	1	1	0	1	CMP_1	Be(2.20, 27.15)	0.04
1	0	1	0	1	CMP_2	Be(1.42, 29.22)	0.01
1	1	1	0	0	CMP_3	Be(1.68, 07.95)	0.09
1	1	0	1	1	CMP_4	Be(1.40, 21.06)	0.02
1	1	0	0	1	CMP_5	Be(1.23, 26.90)	0.01

Table 4.2. The frequency distributions of the significant variables offered to the final Bayesian logistic regression model. Results were based on the analysis of data from 1599 does in 58 Greek dairy goat flocks adjusting for the imperfect Se and Sp of the milk ELISA.

Variable	Category	Milk-ELISA	
		Neg%	Pos%
Origin of the water from surface	No	60.4	5.2
	Yes	30.8	3.6
Contact with other flocks	No	8.0	57.7
	Yes	2.4	31.9
Kids spending hours per day with their does	<10 h	9.1	57.3
	≥10 h	3.5	30.1
Alternating use of antiparasitic compounds	No	37.8	28.4
	Yes	21.6	12.3

Table 4.3. Estimated odds ratios and associated 95% Credible Intervals (CI) for factors associated with the risk of MAP infection after adjusting for the imperfect Se and Sp of the milk ELISA. Results were based on the analysis of data from 1599 does in 58 Greek dairy goats flocks. Flock was included as a random effect.

Variable	Category	Odds ratios (CI)	*P*
Origin of the water from surface	No	1	
	Yes	3.7(1.4;10.4)	0.005
Contact with other flocks	No	1	
	Yes	4.6(1.5;17.4)	0.003
Kids' spending hours per day with their does	<10 h	1	
	≥ 10 h	2.6(1.1;6.4)	0.016
Alternating use of antiparasitic compounds	No	1	
	Yes	0.5(0.2;0.9)	0.020
σ_u^{2} [a]		0.8 (0.1; 2.0)	

[a] the variance of the random effect. u_t . at the flock level.

4.5 Discussion

In this cross-sectional study we found that communal grazing and the use of common water troughs with other flocks was associated with higher odds of MAP infection. This agrees with similar results elsewhere reported, suggesting that contact between flocks is a risk factor for the spread of MAP infection. Mixed farming was a risk factor for caprine paratuberculosis in Jordan (Al-Majali et al., 2008). The only non-infected Chilean dairy goat flocks were the ones that did not import goats from other flocks and were located in geographical areas where no mixed grazing with other susceptible ruminant species took place (Kruze et al., 2007). In Australia, sharing of roads between neighbouring farms was also associated with higher

paratuberculosis infection in sheep flocks (Dhand et al. 2007). Evidently, in areas that are endemically infected with MAP, especially in high agricultural density areas, increased biosecurity measures that would prevent contact between flocks must be part of a control program in order to prevent reintroduction and spread of the same or different MAP strains.

Goats in flocks supplied surface water (from streams or shallow wells) had higher odds to be MAP infected compared to those that were watered by underground and piped water sources. In consistency, an association between lower seroprevalence and presence of piped water was found in a cross- sectional study of small ruminants (Mainar-Jaime and Vazquez-Boland, 1998).However, the access to open water, though believed to aid transmission, was not found to be influential in sheep flocks (Lugton et. al, 2004). Generally open source water is liable to MAP contamination from both domestic and wildlife species. Wildlife could be implicated in paratuberculosis transmission cycles in Greece (Florou et al., 2005). MAP can circulate among wildlife hosts including deer species and rabbits and a possible contamination of the pasture could infect sheep and cattle (Carta et al., 2013). However, MAP excretion by wildlife host is lower than excretion by clinically affected animals (Daniels et al., 2003). Thus, the contamination of the water from the affected goats in the flock should play the major role – compared to contamination due to wildlife – to the spread of MAP infection in endemically infected areas.

Goats in flocks where the kids' were allowed to spend equal to or more than 10 hours per day with their dams had higher odds of MAP infection. Within an infected flock most animals acquire MAP early in their life. Because infection primarily occurs via the fecal oral route, the major source of MAP for the kids is the contaminated with feces udder. Calves that had suckled a foster cow during calfhood had a very high risk of testing ELISA positive compared with calves fed milk replacer indirectly (Nielsen et al, 2008). The direct contact with contaminated milk and colostrum is a major source of MAP infection for suckling ruminants. Under the semi-intensive management system of the Greek dairy flocks, kids directly suckle milk and colostrum from their does. Currently, a program of feeding milk replacement products or pasteurized milk is not applied. Hence, the longer they stay with their dams the more likely they are to ingest higher loads of MAP.

Poor control of intestinal parasites could affect the incidence of paratuberculosis. We found that, the use of the same anti-parasitic compounds rather than the alteration between different anti-parasitic treatments was associated with higher odds of MAP infection. In

consistency to our result, a risk factor study in sheep flocks revealed that the use of ivermectin as the only anti-parasitic treatment was the factor with the strongest association with paratuberculosis seroprevalence (Coelho et al., 2010). Not alternating parasitic treatments or using a single anti-parasitic may contribute to the risk of MAP infection by increasing the probability of goats having higher parasitic loads and enduring longer exposure to parasitic infections. The use of the same antiparasitic compound is associated with increased antiparasitic resistance (Sangster and Gill 1999; Kohler 2001).Further, at the early stages of paratuberculosis, a cell-mediated immune response acts protectively against MAP. A concurrent parasitic infection could cause an easier shift to the humoral immune response (Stabel et al., 2000). However, once this shift occurred, the effect of insufficient antiparasitic treatment in the course of MAP infection is expected to be minimal at the late stages of paratuberculosis (Lugton et al., 2004). The latter authors found no association between the control of parasites and late clinical paratuberculosis in sheep, since drenching of clinical cases simply delayed death. In our study, we adjusted for all the latent stages of infection by incorporating Se and Sp in the models and the observed association primarily concerns the subclinically infected goats because those clinically affected are low yielding animals not maintained for a full lactation period.

A major strength of this study is that we countered the effect of misclassification measured by the imperfect Se and Sp of the milk-ELISA. McInturff et al. (2004) showed that adjusting for the imperfect Se and Sp of the diagnostic process leads to corrected estimates that take into account all latent stages of MAP infection. In our case, we incorporated prior information for the Se and Sp which are based on a recent and relevant validation study for the milk ELISA (Angelidou et al., 2014). Milk ELISA is an imperfect diagnostic test; assuming the opposite would incorporate bias toward to null hypothesis leading to loss of significant variables. Prior information was in the form of probability space rather than single values to capture uncertainty and the analysis was carried out in a flexible Bayesian framework. The cross-sectional nature of the study design has a built-in problem with reverse causation (Martin et al., 2008), i.e. cross-sectional studies capture time-point associations that could not ensure that the animals were not infected prior to the exposure of the identified factors. However, the risk factors in the final model can be considered constant over time since they represent either routine managerial practices. This minimizes the limitations arising from the cross-sectional design. Another likely study limitation is the inflation of the Type I error rate due to multiple

hypothesis testing, the consequence of testing the association with outcome of numerous variables (Kleinbaum 1994). The paucity of previous similar studies on goats made necessary the development of a rather detailed questionnaire with many factors. This concern is, however, restricted by the somewhat strong associations ($0.003 < p < 0.02$) in the final model.

In conclusion, the use of common water troughs, communal grazing, surface water and kids' spending equal to or more than 10 hours per day with their dams were associated with higher odds of MAP infection. Finally, the alternating use of different anti-parasitic compounds was associated with lower odds of MAP infection. These results should be considered in the development of a nationwide future control program for caprine paratuberculosis in Greece.

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Chapter 5

5. General discussion and future perspectives

Greece has almost 10 million dairy sheep and 6 million dairy goats in flocks reared across its mainland and islands. Infection with MAP is widely distributed. The animals are primarily reared for milk production which is used for the production of several types of cheese. At least 30% of those are exported. Recent research recovered MAP or detected MAP DNA in several cheeses at retail. A potential zoonotic link between MAP and human inflammatory bowel diseases including Crohn's disease has been suggested but remains unclear. If MAP is confirmed as a zoonotic pathogen, public confidence in products of the Greek small ruminant industry is very likely to decline. In other countries exporting products mainly from dairy cows national control programs to control MAP infection have been developed and implemented. Therefore there is a need for development of a program to reduce the level of MAP infection in Greek sheep and goats. The program should be tailored to the average general management of Greek flocks without increasing the cost of production.

Diagnostic tests are the backbones of control programs against MAP. They are not only used to define targets and assess progress but give the basis for managing the risk of MAP spread at the herd/flock or area level. Diagnostic tests against MAP should be ideally validated in the animal population they are going to be applied since their performance varies among animal species and is affected by population specific characteristics. For a national control program, tests with acceptable diagnostic accuracy which can give quick results at relatively low sample cost may be preferred to accurate but expensive ones that require time consuming sample processing. In this sense, the serological tests, which fall in the former group of tests, and especially the ELISAs which can be automated, may be preferred over agent detecting tests, such as culture or PCR, which fall in the latter group of tests. However, their limitations should be appraised before their use in the control program. In the past we have evaluated the performance of a serological indirect ELISA in Greek sheep and goats. Compared to serum milk, however, is easily accessible sample which can be collected from live animals at a fraction of the cost of serum collection, because it is obtained by farmers and not by veterinarians repeatedly for testing for several reasons. Evidently, the milk ELISA has many advantages for becoming at least one of the tests of choice for a national control program against MAP infection in Greek

sheep and goats. Milk sampling and testing is central to control programs against MAP infection in dairy cattle in other European countries.

Since it is wrong to extrapolate results from dairy cattle to sheep and goats, in this thesis we evaluated the diagnostic validity of a commercially available milk ELISA separately in dairy sheep and goats. In dairy cows a variation in the milk antibody trend across lactation was detected. Thus, we performed a lactation stage specific validation by applying non gold standard methodology in Bayesian mixture models adjusting for the potential within animal and within lactation stage correlation.

In goats, we found that both serum- and milk-ELISA, in all lactation stages (at kidding, early, mid and late lactation) were of average to high overall discriminating ability as measured by the AUC. Both tests had comparable AUCs across the different lactation stages. Further, for either test, there was not a significant difference between the different lactation stages with the exception of the estimated AUC for the milk-ELISA during kidding that had a lower mean value of 63% (95%CrIs 39; 82). For both tests, the highest power to discriminate healthy from infected does was at late lactation. When the cutoff values recommended by the manufacturer were decreased by 50%, the Ses were increased without serious loss of Sps. Lastly, we showed that the estimates obtained from the model assuming distinct prevalence for each lactation stage and the one with constant prevalence were comparable, indicating that the non-lactation stage-specific prevalence was similar. In sheep, both tests in all lactation stages were of low to moderate overall discriminating ability and had comparable AUCs across the different lactation stages which did not differ between the different lactation stages. When the cutoff values recommended by the manufacturer were decreased by 50%, the Ses were increased without serious loss of Sps.

Since the milk-ELISA had acceptable diagnostic accuracy only in goats we used this test in our attempt to identify flock characteristics associated with increased MAP infection in a cross-sectional study of goat flocks in a region of Central Greece with significant goat farming. The outcome of this study is important for the development of a national control risk-based program with a framework for identification of high risk, for the spread of MAP infection, flocks and regions. We sampled the flocks at late lactation and interpreted the results of the milk-ELISA in the previously optimized cutoffs. We analysed the data in a Bayesian framework adjusting for the imperfect Se and Sp of the test. The prior information incorporated for the Se and Sp were

based on our previous validation study. We found that communal grazing and the use of common water troughs with other flocks was associated with higher odds of MAP infection. Evidently, in areas that are endemically infected with MAP, especially in high agricultural density areas, increased biosecurity measures that would prevent contact between flocks must be part of a control program in order to prevent reintroduction and spread of the same or different MAP strains. Goats in flocks supplied surface water (from streams or shallow wells) had higher odds to be MAP infected compared to those that were watered by underground and piped water sources. Generally open source water is liable to MAP contamination from both domestic and wildlife species. Goats in flocks were the kids' were allowed to spend equal to or more than 10 hours per day with their dams had higher odds of MAP infection. Within an infected flock most animals acquire MAP early in their life. Because infection primarily occurs via the fecal oral route, the major source of MAP for the kids is the contaminated with feces udder. The direct contact with contaminated milk and colostrum is a major source of MAP infection for suckling ruminants. Under the semi-intensive management system of the Greek dairy flocks, kids directly suckle milk and colostrum from their does. Currently, a program of feeding milk replacement products or pasteurized milk is not applied. Hence, the longer they stay with their dams the more likely they are to ingest higher loads of MAP. Poor control of intestinal parasites affected the odds of infection. The use of the same anti-parasitic compound rather than the alteration between different anti-parasitic treatments was associated with higher odds of MAP infection. Not alternating parasitic treatments or using a single anti-parasitic may contribute to the risk of MAP infection by increasing the probability of goats having higher parasitic loads and enduring longer exposure to parasitic infections. The use of the same antiparasitic compound is associated with increased antiparasitic resistance.

In conclusion, we showed that the milk-ELISA is a test with acceptable diagnostic accuracy in goats but not in sheep. It can be used in a national control program against MAP infection of only goats. Other tests, probably antigen detecting ones, may be preferable for sheep. Other tests, probably antigen detecting ones, may be preferable for sheep. For Greek goat flocks, it should be anticipated that the prevalence and incidence of MAP-infection would be highest in regions with communal watering and grazing. In these regions shallow water wells are a likely source of infection to the animals. Management of the kids and strategically designed antiparasitic treatments should be parts of an integrated control program.

Appendix

Appendix 1.1. WinBugs code for the estimation of the of the Area Uner the Curve, the Sensitivity and Specificity at several cutoffs, for two correlated tests for four repeated measurements without a Gold Standard assuming four prevalences for each lactation stage

```
model{

for (i in 1:N){
# the log-normal S/P values for each stage of lactation
Kid[i,1:K]~dmnorm(mu1[D1[i],], Omega1[D1[i],,])
Early[i,1:K]~dmnorm(mu2[D2[i],], Omega2[D2[i],,])
Mid[i,1:K]~dmnorm(mu3[D3[i],], Omega3[D3[i],,])
Late[i,1:K]~dmnorm(mu4[D4[i],], Omega4[D4[i],,])

D1[i]~ dcat(PKid[])
D2[i]~ dcat(PEarly[])
D3[i]~ dcat(PMid[])
D4[i]~ dcat(PLate[])
}
#the prevalence of the healthy and the MAP infected at each stage of lactation
PKid[1:2]~ddirch(alpha1[])
PEarly[1:2]~ddirch(alpha2[])
PMid[1:2]~ddirch(alpha3[])
```

PLate[1:2]~ddirch(alpha4[])

for (j in 1:K){

#the mean value of the healthy and the infected during kidding

mu1[1,j] <- beta1[1,j]

mu1[2,j] <- beta1[2,j]

beta1[1,j]~dnorm(0, 0.001)

beta1[2,j]~dnorm(0, 0.001)

#the mean value of the healthy and the infected at the early lactation stage

mu2[1,j] <- beta2[1,j]

mu2[2,j] <- beta2[2, j]

beta2[1,j]~dnorm(0, 0.001)

beta2[2,j]~dnorm(0, 0.001)

#the mean value of the healthy and the infected at the mid lactation stage

mu3[1, j] <- beta3[1, j]

mu3[2, j] <- beta3[2, j]

beta3[1, j]~dnorm(0, 0.001)

beta3[2, j]~dnorm(0, 0.001)

#the mean value of the healthy and the infected at the late lactation stage

mu4[1, j] <- beta4[1, j]

mu4[2, j] <- beta4[2, j]

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```
beta4[1,j]~dnorm(0, 0.001)

beta4[2,j]~dnorm(0, 0.001)

}


Omega1[1, 1:K, 1:K] ~dwish(R11[,], 2)

Omega1[2, 1:K, 1:K] ~dwish(R12[,], 2)

Omega2[1, 1:K, 1:K] ~dwish(R21[,], 2)

Omega2[2, 1:K, 1:K] ~dwish(R22[,], 2)

Omega3[1, 1:K, 1:K] ~dwish(R31[,], 2)

Omega3[2, 1:K, 1:K] ~dwish(R32[,], 2)

Omega4[1, 1:K, 1:K] ~dwish(R41[,], 2)

Omega4[2, 1:K, 1:K] ~dwish(R42[,], 2)

Sigma1[1, 1:K, 1:K] <- inverse(Omega1[1, 1:K, 1:K])

Sigma1[2, 1:K, 1:K] <- inverse(Omega1[2, 1:K, 1:K])

Sigma2[1, 1:K, 1:K] <- inverse(Omega2[1, 1:K, 1:K])

Sigma2[2, 1:K, 1:K] <- inverse(Omega2[2, 1:K, 1:K])

Sigma3[1, 1:K, 1:K] <- inverse(Omega3[1, 1:K, 1:K])

Sigma3[2, 1:K, 1:K] <- inverse(Omega3[2, 1:K, 1:K])

Sigma4[1, 1:K, 1:K] <- inverse(Omega4[1, 1:K, 1:K])

Sigma4[2, 1:K, 1:K] <- inverse(Omega4[2, 1:K, 1:K])
```

```
#estimation of the Area Under the Curve

for (l in 1:8){

AUC1.ph[l] <- phi(-(beta1[1,l] - beta1[2,l])/sqrt(Sigma1[1,l,l] + Sigma1[2,l,l]))

AUC2.ph[l] <- phi(-(beta2[1,l] - beta2[2,l])/sqrt(Sigma2[1,l,l] + Sigma2[2,l,l]))

AUC3.ph[l] <- phi(-(beta3[1,l] - beta3[2,l])/sqrt(Sigma3[1,l,l] + Sigma3[2,l,l]))

AUC4.ph[l] <- phi(-(beta4[1,l] - beta4[2,l])/sqrt(Sigma4[1,l,l] + Sigma4[2,l,l]))

for (k in 1:2){

Replicate1[k,l]~ dnorm(mu1[k,l] , Omega1[k,l,l])

Replicate2[k,l]~ dnorm(mu2[k,l] , Omega1[k,l,l])

Replicate3[k,l]~ dnorm(mu3[k,l] , Omega1[k,l,l])

Replicate4[k,l]~ dnorm(mu4[k,l] , Omega1[k,l,l])

}

# Sensitivity & Specificity in Sera of the serum ELISA at the recommended & 50% reduced cutoffs

for (s in 1:4){

# during kidding
```

```
c11.ph.rec[s]<-((0.37)-beta1[2,s])/sqrt(Sigma1[2,s,s])

Se1.ph.rec[s]<- 1-phi(c11.ph.rec[s])

c21.ph.rec[s]<-((0.37)-beta1[1,s])/sqrt(Sigma1[1,s,s])

Sp1.ph.rec[s]<-phi(c21.ph.rec[s])

c11.ph.red[s]<-((0.20)-beta1[2,s])/sqrt(Sigma1[2,s,s])

Se1.ph.red[s]<- 1-phi(c11.ph.red[s])

c21.ph.red[s]<-((0.20)-beta1[1,s])/sqrt(Sigma1[1,s,s])

Sp1.ph.red[s]<-phi(c21.ph.red[s])

# early lactation stage

c12.ph.rec[s]<-((0.37)-beta2[2,s])/sqrt(Sigma2[2,s,s])

Se2.ph.rec[s]<- 1-phi(c12.ph.rec[s])

c22.ph.rec[s]<-((0.37)-beta2[1,s])/sqrt(Sigma2[1,s,s])

Sp2.ph.rec[s]<-phi(c22.ph.rec[s])

c12.ph.red[s]<-((0.20)-beta2[2,s])/sqrt(Sigma2[2,s,s])

Se2.ph.red[s]<- 1-phi(c12.ph.red[s])

c22.ph.red[s]<-((0.20)-beta2[1,s])/sqrt(Sigma2[1,s,s])

Sp2.ph.red[s]<-phi(c22.ph.red[s])

# mid lactation stage

c13.ph.rec[s]<-((0.37)-beta3[2,s])/sqrt(Sigma3[2,s,s])

Se3.ph.rec[s]<- 1-phi(c13.ph.rec[s])

c23.ph.rec[s]<-((0.37)-beta3[1,s])/sqrt(Sigma3[1,s,s])
```

```
Sp3.ph.rec[s]<-phi(c23.ph.rec[s])

c13.ph.red[s]<-((0.20)-beta3[2,s])/sqrt(Sigma3[2,s,s])

Se3.ph.red[s]<- 1-phi(c13.ph.red[s])

c23.ph.red[s]<-((0.20)-beta3[1,s])/sqrt(Sigma3[1,s,s])

Sp3.ph.red[s]<-phi(c23.ph.red[s])

# late lactation stage

c14.ph.rec[s]<-((0.37)-beta4[2,s])/sqrt(Sigma4[2,s,s])

Se4.ph.rec[s]<- 1-phi(c14.ph.rec[s])

c24.ph.rec[s]<-((0.37)-beta4[1,s])/sqrt(Sigma4[1,s,s])

Sp4.ph.rec[s]<-phi(c24.ph.rec[s])

c14.ph.red[s]<-((0.20)-beta4[2,s])/sqrt(Sigma4[2,s,s])

Se4.ph.red[s]<- 1-phi(c14.ph.red[s])

c24.ph.red[s]<-((0.20)-beta4[1,s])/sqrt(Sigma4[1,s,s])

Sp4.ph.red[s]<-phi(c24.ph.red[s])

}


# Sensitivity  & Specificity of the milk ELISA at the recommended & 50% reduced cutoffs


for (m in 5:8){

# during kidding

c11.ph.rec[m]<-((0.18)-beta1[2,m])/sqrt(Sigma1[2,m,m])
```

```
Se1.ph.rec[m]<- 1-phi(c11.ph.rec[m])

c21.ph.rec[m]<-((0.18)-beta1[1,m])/sqrt(Sigma1[1,m,m])

Sp1.ph.rec[m]<-phi(c21.ph.rec[m])

c11.ph.red[m]<-((0.10)-beta1[2,m])/sqrt(Sigma1[2,m,m])

Se1.ph.red[m]<- 1-phi(c11.ph.red[m])

c21.ph.red[m]<-((0.10)-beta1[1,m])/sqrt(Sigma1[1,m,m])

Sp1.ph.red[m]<-phi(c21.ph.red[m])

# early lactation stage

c12.ph.rec[m]<-((0.18)-beta2[2,m])/sqrt(Sigma2[2,m,m])

Se2.ph.rec[m]<- 1-phi(c12.ph.rec[m])

c22.ph.rec[m]<-((0.18)-beta2[1,m])/sqrt(Sigma2[1,m,m])

Sp2.ph.rec[m]<-phi(c22.ph.rec[m])

c12.ph.red[m]<-((0.10)-beta2[2,m])/sqrt(Sigma2[2,m,m])

Se2.ph.red[m]<- 1-phi(c12.ph.red[m])

c22.ph.red[m]<-((0.10)-beta2[1,m])/sqrt(Sigma2[1,m,m])

Sp2.ph.red[m]<-phi(c22.ph.red[m])

# mid lactation stage

c13.ph.rec[m]<-((0.18)-beta3[2,m])/sqrt(Sigma3[2,m,m])

Se3.ph.rec[m]<- 1-phi(c13.ph.rec[m])

c23.ph.rec[m]<-((0.18)-beta3[1,m])/sqrt(Sigma3[1,m,m])

Sp3.ph.rec[m]<-phi(c23.ph.rec[m])
```

```
c13.ph.red[m]<-((0.10)-beta3[2,m])/sqrt(Sigma3[2,m,m])

Se3.ph.red[m]<- 1-phi(c13.ph.red[m])

c23.ph.red[m]<-((0.10)-beta3[1,m])/sqrt(Sigma3[1,m,m])

Sp3.ph.red[m]<-phi(c23.ph.red[m])

# late lactation stage

c14.ph.rec[m]<-((0.18)-beta4[2,m])/sqrt(Sigma4[2,m,m])

Se4.ph.rec[m]<- 1-phi(c14.ph.rec[m])

c24.ph.rec[m]<-((0.18)-beta4[1,m])/sqrt(Sigma4[1,m,m])

Sp4.ph.rec[m]<-phi(c24.ph.rec[m])

c14.ph.red[m]<-((0.10)-beta4[2,m])/sqrt(Sigma4[2,m,m])

Se4.ph.red[m]<- 1-phi(c14.ph.red[m])

c24.ph.red[m]<-((0.10)-beta4[1,m])/sqrt(Sigma4[1,m,m])

Sp4.ph.red[m]<-phi(c24.ph.red[m])}}}
```

Appendix 1.2. WinBugs code for the estimation of the of the Area Uner the Curve, the Sensitivity and Specificity at several cutoffs, for two correlated tests for four repeated measurements without a Gold Standard assuming constant prevalence across lactation.

```
model{

for (i in 1:N){
Response[i,1:K]~dmnorm(mu[D[i],1:K], Omega[D[i], ,])
D[i] ~dcat(P[]) #the latent variable}

P[1:2]~ddirch(alpha[])

for (j in 1:K){
mu[1,j] <- beta[1,j]#mean vaues of the healthy
mu[2,j] <- beta[2,j]#mean values of the MAP infected
beta[1,j]~dnorm(0, 0.001)
beta[2,j]~dnorm(0, 0.001) }

Omega[1, 1:K, 1:K] ~dwish( R1[,], 8)
Omega[2, 1:K, 1:K] ~dwish( R2[,], 8)
Sigma[1, 1:K, 1:K] <- inverse(Omega[1, 1:K, 1:K])
Sigma[2, 1:K, 1:K] <- inverse(Omega[2, 1:K, 1:K])

for (i in 1:8){
for (j in 1:8){
rho[1,i,j]<- Sigma[1,i,j]/sqrt(Sigma[1,i,i]*Sigma[1,j,j])
rho[2,i,j]<- Sigma[2,i,j]/sqrt(Sigma[2,i,i]*Sigma[2,j,j])}}

#estimation of the Area Under the Curve
```

```
for (l in 1:8){
AUC.ph[l] <- phi(-(beta[1,l] - beta[2,l])/sqrt(Sigma[1,l,l] + Sigma[2,l,l]))
for (k in 1:2){
Replicate[k,l]~ dnorm(mu[k,l] , Omega[k,l,l])}

for (i in 1:100){# Sensitivity and Specificity at several cutoffs
c1[l,i]<-((-0.5+ 0.02*i)-beta[2,l])/sqrt(Sigma[2,l,l])
Se.ph[l,i]<- 1-phi(c1[l,i])
c2[l,i]<-((-0.5+ 0.02*i)-beta[1,l])/sqrt(Sigma[1,l,l])
Sp.ph[l,i]<-phi(c2[l,i])
# to estmimate the Youden's idex
S[l,i]<- Se.ph[l,i] + Sp.ph[l,i] - 1
c[i] <- (-0.5+ 0.02*i)}}

# Sensitivity  & Specificity of the Serum ELISA at the recommended & 50% reduced cutoffs

for (s in 1:4){
c1.ph.rec[s]<-((0.37)-beta[2,s])/sqrt(Sigma[2,s,s])
Se.ph.rec[s]<- 1-phi(c1.ph.rec[s])
c2.ph.rec[s]<-((0.37)-beta[1,s])/sqrt(Sigma[1,s,s])
Sp.ph.rec[s]<-phi(c2.ph.rec[s])
c1.ph.red[s]<-((0.20)-beta[2,s])/sqrt(Sigma[2,s,s])
Se.ph.red[s]<- 1-phi(c1.ph.red[s])
c2.ph.red[s]<-((0.20)-beta[1,s])/sqrt(Sigma[1,s,s])
Sp.ph.red[s]<-phi(c2.ph.red[s])}

# Sensitivity  & Specificity of the Milk ELISA at the recommended & 50% reduced cutoffs

for (m in 5:8){
c1.ph.rec[m]<-((0.18)-beta[2,m])/sqrt(Sigma[2,m,m])
Se.ph.rec[m]<- 1-phi(c1.ph.rec[m])
```

```
c2.ph.rec[m]<-((0.18)-beta[1,m])/sqrt(Sigma[1,m,m])
Sp.ph.rec[m]<-phi(c2.ph.rec[m])
c1.ph.red[m]<-((0.10)-beta[2,m])/sqrt(Sigma[2,m,m])
Se.ph.red[m]<- 1-phi(c1.ph.red[m])
c2.ph.red[m]<-((0.10)-beta[1,m])/sqrt(Sigma[1,m,m])
Sp.ph.red[m]<-phi(c2.ph.red[m])}}
```

Appendix 1.3.WinBugs code for the estimation of the of the Area Uner the Curve, the Sensitivity and Specificity at several cutoff values, for two correlated tests for four repeated measurements without a Gold Standard assuming constant prevalence across lactation and implementing prior information.

```
model{

for (i in 1:N){
R[i,1:K]~dmnorm(mu[D[i],1:K], Omega[D[i], ,])
D[i] ~dcat(P[])}

P[1:2]~ddirch(alpha[])

for (j in 1:K){
mu[1,j] <- beta[1,j]
mu[2,j] <- beta[2,j]
# prior information on the mean value of the MAP infected and the healthy
beta[1,j]~dbeta(2, 5) #95% sure less than 0.8 and mode at 0.3
beta[2,j]~dbeta(1.5, 54.3)} # 99% sure less than 0.1 mode at 0.01

Omega[1, 1:K, 1:K] ~dwish( R1[,], 8)
Omega[2, 1:K, 1:K] ~dwish( R2[,], 8)
Sigma[1, 1:K, 1:K] <- inverse(Omega[1, 1:K, 1:K])
Sigma[2, 1:K, 1:K] <- inverse(Omega[2, 1:K, 1:K])

for (i in 1:8){
for (j in 1:8){
rho[1,i,j]<- Sigma[1,i,j]/sqrt(Sigma[1,i,i]*Sigma[1,j,j])
rho[2,i,j]<- Sigma[2,i,j]/sqrt(Sigma[2,i,i]*Sigma[2,j,j])}}
```

```
for (l in 1:8){
AUC.ph[l] <- phi((beta[1,l] - beta[2,l])/sqrt(Sigma[1,l,l] + Sigma[2,l,l]))
for (k in 1:2){
Replicate[k,l]~ dnorm(mu[k,l] , Omega[k,l,l])}
#grid line is for -1,52 until 1,9
for (i in 1:170){
c1[l,i]<-((-1.5+ 0.02*i)-beta[1,l])/sqrt(Sigma[1,l,l])
Se.ph[l,i]<- 1-phi(c1[l,i])
c2[l,i]<-((-1.5+ 0.02*i)-beta[2,l])/sqrt(Sigma[2,l,l])
Sp.ph[l,i]<-phi(c2[l,i])}}

# Sensitivity  & Specificity of the Serum ELISA at the recommended & 50% reduced cutoffs

for (s in 1:4){
c1.ph.rec[s]<-((0.43)-beta[1,s])/sqrt(Sigma[1,s,s])
Se.ph.rec[s]<- 1-phi(c1.ph.rec[s])
c2.ph.rec[s]<-((0.43)-beta[2,s])/sqrt(Sigma[2,s,s])
Sp.ph.rec[s]<-phi(c2.ph.rec[s])
c1.ph.red[s]<-((0.28)-beta[1,s])/sqrt(Sigma[1,s,s])
Se.ph.red[s]<- 1-phi(c1.ph.red[s])
c2.ph.red[s]<-((0.28)-beta[2,s])/sqrt(Sigma[2,s,s])
Sp.ph.red[s]<-phi(c2.ph.red[s])}

# Sensitivity & Specificity of the milk ELISA at the recommended & 50% reduced cutoffs

for (m in 5:8){

c1.ph.rec[m]<-((0.25)-beta[1,m])/sqrt(Sigma[1,m,m])
Se.ph.rec[m]<- 1-phi(c1.ph.rec[m])
c2.ph.rec[m]<-((0.25)-beta[2,m])/sqrt(Sigma[2,m,m])
```

```
Sp.ph.rec[m]<-phi(c2.ph.rec[m])
c1.ph.red[m]<-((0.15)-beta[1,m])/sqrt(Sigma[1,m,m])
Se.ph.red[m]<- 1-phi(c1.ph.red[m])
c2.ph.red[m]<-((0.15)-beta[2,m])/sqrt(Sigma[2,m,m])
Sp.ph.red[m]<-phi(c2.ph.red[m])}}
```

Appendix 2.WinBugs code for the Bayesian logistic regression model that adjusted for imperfect Se and Sp of the diagnostic test.

model

{

for (i in 1:N){ # where r is the number of positive does

r[i] ~ dbin(Ap[i],n[i]) # Incorporation of test sensitivity and specificity

Ap[i] < −Se*Tp[i] + (1 − Sp)*(11 − Tp[i]) logit(Tp[i]) < −b[1] + b[2]*X1[i] + b[3]*X2[i] + b[4]*X3[i] + b[5]*X4[i] + u[i] }

Informative priors on sensitivity and specificity

Sp ~dbeta(315.32, 1.62) Se ~ dbeta(20.3,10.08) tau~dgamma(1.0E-3, 1.0E-3) sigma < −1⁄sqrt(tau) sigma2 < −1⁄tau

p[1] ~ dbeta(2.20, 27.15) p[2] ~ dbeta(1.42, 29.22) p[3] ~ dbeta(1.68, 7.95) p[4] ~ dbeta(1.40, 21.06) p[5] ~ dbeta(1.23, 26.9)

#Conditional mean priors specification

b[1] < −xinv[1,1]*logit(p[1]) + xinv[1,2]*logit(p[2]) + xinv[1,3]*logit(p[3]) + [1,4]*logit(p[4]) + xinv[1,5]*logit(p[5])

b[2] < −xinv[2,1]*logit(p[1]) + xinv[2,2]*logit(p[2]) + xinv[2,3]*logit(p[3]) + xinv[2,4]*logit(p[4]) + xinv[2,5]*logit(p[5])

b[3] < −xinv[3,1]*logit(p[1]) + xinv[3,2]*logit(p[2]) + xinv[3,3]*logit(p[3]) + xinv[3,4]*logit(p[4]) + xinv[3,5]*logit(p[5])

b[4] < −xinv[4,1]*logit(p[1]) + xinv[4,2]*logit(p[2]) + xinv[4,3]*logit(p[3]) + xinv[4,4]*logit(p[4]) + xinv[4,5]*logit(p[5])

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b[5]xinv[5,1]*logit(p[1]) + xinv[5,2]*logit(p[2]) + xinv[5,3]*logit(p[3]) + xinv[5,4]*logit(p[4]) + xinv[5,5]*logit(p[5])

for(j in 1:5){ P[j] < −step(b[j]) #computation of odds

Odd[j] < −exp(x[1,j]*b[1] + x[2,j]*b[2] + x[3,j]*b[3] + x[4,j]*b[4] + x[5,j]*b[5]) }}

Appendix 3.Questionnaire administrated to the the farmers for the investigation of risk factors affecting the spread of Paratuberculosis.

QUESTIONNAIRE

RISK FACTORS AFFECTING THE SPREAD OF PARATUBERCULOSIS IN GREEK DAIRY GOAT FLOCKS

FARMER CONTACT DETAILS

LA N LAST NAME _____FIRST NAME _____

POS POSTAL ADDRESS _____

PHONE_____

GENERAL QUESTIONS ABOUT THE FARM

Q-1) How many animals are today in your flock?

DOES_____

BUCKS_____

KIDS (born in this year)_____

Q-2) Transhumance?

1. YES
2. NO

Q-3) If you answered “yes” above, fill in the following:

DEPARTURE DATE FROM THE WINTER SHED

_____

RETURN DATE TO THE WINTER

SHED_____

REGION SPENT

DURINGSUMMER_____

Q-4) What is the size of the farm (number of sheds, yard)?

Winter	Summer
1. _____ m^2 SHED	1. _____ m^2 SHED
2. _____ m^2 SHED	2. _____ m^2 SHED
3. _____ m^2 SHED	3. _____ m^2 SHED
4. _ _____m^2 SHED	4. _ _____m^2 SHED
5. _____ m^2 YARD	5. _____ m^2 YARD

STOCKING DENSITY DURING WINDER: _____ANIMALS/m^2

STOCKING DENSITY DURING SUMMER: _____ANIMALS/m^2

Q-5) Give some details of the winter shed construction:

HEIGHT (m):_____

KIND OF ROOF:_____

KIND OF WALL:_____

FLOORING:_____

VENTILATION:_____

MILKING MACHINE(Y/N):_____

Q-6) Describe the shed location:

Summer

SLOPE: _____ _____

ALTITUDE: _____

ORIENTATION: _____

DISTANCE FROM INHABITED AREA:

_____

Winter

SLOPE: _____ _____

ALTITUDE: _____

ORIENTATION: _____

DISTANCE FROM INHABITED AREA:

_____

Q-7) Percentage of the following breeds in your flock.

1. INDIGENOUS_____
2. ALPINE_____
3. SCOPELOU_____
4. OTHER(SPECIFY)_____
5. CROSSES (SPECIFY) _____

Q-8) How many bucks from other flocks you borrowed during the mating season?

_____

_____

Q-9) When (month) did early and late kidding begun?

EARLY:_____

LATE: _____

WATER SUPPLY

Q-10) Origin of the water for the animals

1. STREAM
2. UNDERGROUND
3. WATER PONDS
4. PIPED
5. OTHER(specify)_____

Q-11) If you pipe water into the water troughs, what is the main source during winter?

1. SURFACE WATER (STREAM)
2. UNDERGROUND (WELL, SPRING, BORE)
3. OTHER(SPECIFY)_____

Q-12) If you pipe water into the water troughs, what is the main source during summer?

1. SURFACE WATER (STREAM)
2. UNDERGROUND (WELL, SPRING, BORE)
3. OTHER(SPECIFY)_____

Q-13) Kind of the water troughs in the shed:

1. GROUPED
2. INDIVIDUAL
3. BOTH

Q-14)The water troughs in the shed are made of

1. PLASTIC
2. WOOD
3. STAINLESS STEAL
4. OTHER (SPECIFY)_____

Q-15) Number, length and width of the troughs in the shed?

_____

_____

_____

EXPOSURE OF THE KID POST PARTUM

Q-16) Where do the early kidding does usually deliver? (Rank the answers below starting from the the most possible)

1. GRAZING AREA ☐
2. IN THE SHED SEPARATE FROM OTHER DOES ☐
3. IN THE YARD ☐
4. IN THE SHED WITH OTHER DOES ☐

Q-17) Where do the late kidding does usually deliver? (Rank the answers below starting from the the most possible)

1. GRAZING AREA/YARD ☐
2. IN THE SHED SEPARATELY FROM OTHER DOES ☐
3. IN THE SHED WITH OTHER DOES ☐
4. IN SEPARATE SHED ☐

Q-18) If the does deliver in the shed separate from other does, which is the size of the kidding area and how many does do you stock?

STOCKING DENSITY: _____m^2/_____ DOES

Q-19) Which is the material used to separate the maternity paddock from the rest of the shed?

1. FENCE, WITH THE ABILITY TO BE REMOVED
2. FENCE, WITHOUT THE ABILITY TO BE REMOVED
3. SEPARATE SHED

Q-20) Do you add bedding material in the maternity paddock?

1. STRAW
2. SAWDUST

3. NO
4. OTHER (SPECIFY)_____

Q-21) How do you prefer to clean the maternity paddock after each group delivery?

1. ADD STRAW
2. APPLY DISINFECTANT
3. OTHER_____

Q-22) How often do you clean the maternity paddock?

1. ALWAYS(AFTER EACH GROUP DELIVERY)
2. OFTEN(EVERYDAY)
3. OCCASIONALLY (EVERY 10 DAYS)
4. RARELY (EVERY MONTH)

Q-23) If you use disinfectant in the maternity paddock, describe:

COMMERCIAL NAME_____

DOSE_____ _____ __

DURATION APPLIED_____

Q-24) After kidding is the maternity paddock used for other purposes? (e.g. isolation of sick animals, bucks housing)

1. YES
2. NO

EXPOSURE OF THE KIDS DURING SUCKLING

Q-25) Do you let the kids milk suckle their does?

1. YES
2. NO
3. YES BUT SOME KIDS ARE RAISED BY HAND-FEEDING

Q-26) If there are kids that did not suckle their does, the percentage is

1. EQUAL TO OR BELOW 2%
2. 5%
3. MORE THAN 5%

Q-27) Which is the origin of the colostrum /milk you used to feed the kids that did not suckle their does?

1. FOSTER DOE
2. OTHER (SPECIFY)_____

Q-28) Which percentage of the kids is usually fostered?

_____%

Q-29) If you raised the kids by hand-feeding, how many days did you allow the newborns to suckle colostrum/milk from their does?

1. LESS THAN 3 DAYS
2. 3 TO 5 DAYS
3. MORE THAN 5 DAYS

Q-30) How many hours per day do the kids spend with their does until weaning?

1. LESS THAN 10

2. MORE THAN OR EQUAL TO 10

Q-31) Do you usually clean the teats of the does before sucking?

1. YES
2. NO

Q-32)Do the kids and the goats share food during pre-weaning?

1. YES
2. NO

Q-33)Do the kids and the goats share water during pre-weaning?

1. YES
2. NO

PRODUCTION PARAMETERS: CULLING AND DEATH RATE

Q-34) Percentage of adult animals culled or died yearly in the last five years?

(Specify the main reason of deaths):

_____

_____

_____

Q-35) When do you usually cull for age?

1. UNDER 4 YEARS
2. 5 YEARS
3. 6 YEARS
4. 7 YEARS OR OLDER

BIOSECURITY

Q-36) How many animals did you purchased last year?

_____

Q- 37) How many kids did you kept for replacements last year?

_____

Q-38) Which was the paratuberculosis infection status of the flocks from which you purchased animals from;

1. WITH HISTORY OF PARATUBERCULOSIS
2. UNKNOWN HISTORY OF PARATUBERCULOSIS
3. FREE FROM PARATUBERCULOSIS

Q-39) Do you keep other animals in your farm (poultry, pigs, horses, etc)?

SPECIES: NUMBER

1. _____ : _____
2. _____ : _____
3. _____ : _____
4. _____ : _____
5. _____ : _____

Q-40) Does your flock share pasture and water troughs with other flocks/herds?

1. YES

2. NO

Q-41) Type and number of the flocks/herds sharing pasture and water troughs?

1. SHEEP_____
2. GOAT_____
3. MIXED(SHEEP AND GOAT)_____
4. CATTLE_____
5. OTHER (SPECIFY)_____

Q-42) Is there any contact with wildlife?

	YES	NO
3. DURING GRAZING	☐	☐
4. WATER SUPPLYING	☐	☐
5. USE OF COMMON ROADS	☐	☐

Q-43) Wildlife having contact with the flock (Specify in decreasing frequency)

_____
_____

GASTROINTESTINAL PARASITE CONTROL

Q-44) When did the last control for the gastrointestinal parasites occurred?

DATE_____
BREEDING STAGE_____

Q-45) which treatments did you apply the past five years to control gastrointestinal parasites?

BRAND NAMES_____

DOSES_____

____

Q-46) Type of formulation

1. TABS
2. ORAL SUSPENSION
3. INJECTION

Q-47) How often do you alter the use of anti-parasitic compounds?

1. NEVER
2. EVERY FIVE YEARS
3. EVERY TWO YEARS
4. YEARLY

Q-48) In which combination were the anti-parasitic compounds used?

1. ONLY IVERMECTIN
2. ONLY LEVAMIZOLE
3. CHOICE ONE OR TWO COMBINED WITH RAFOXANIDE
4. BENZIMIDAZOLE
5. OTHER(SPECIFY)_____

Q-49) Did you apply any treatment to prevent occurrence of neonatal coccidiosis?

1. YES
2. NO

Q-50) Which vaccinations are applied in the flock?

1. PARATUBERCULOSIS
2. CLOSTRIDIAL
3. CONTAGIOUS AGALATIA
4. PASTEURELLOSIS
5. COLLIBACILOSIS
6. CHLAMYDIOSIS
7. OTHER(SPECIFY)_____

NUTRITION

Q-51) When providing additional feed in the shed, which type of concentrates (grain) are most commonly provided during winter?

_____
_____

Q-52) When hand feeding in the shed, which type of concentrates (grain) are most commonly provided during summer?

_____
_____

Q-53) When providing additional feed in the shed, which type of bulk food (hay, silage) are most commonly provided during winter?

_____
_____

Q-54) When hand feeding in the shed, which type of bulk food (hay, silage) are most commonly provided during summer?

_____
_____

Q-55) How many hours per day did your flock spend on pasture during winter?

1. LESS THAN 5 HOURS
2. FIVE HOURS OR MORE

Q-56) How many hours per day did your flock spend on pasture during summer?

1. LESS THAN 7 HOURS
2. 7 HOURS OR MORE

Q-57) When additional concentrates are fed to the flock, do you give supplements containing minerals?

	YES	NO
1. TO THE KIDS DURING PRE-WEANING	☐	☐
2. TO THE DOES AT THE EARLY STAGE OF LACTATION	☐	☐
3. TO THE DOES AT THE MID STAGE OF LACTATION	☐	☐
4. TO THE DOES AT THE LATE STAGE OF LACTATION	☐	☐
5. DURING THE MATING SEASON	☐	☐

Q-58) If you give supplements containing minerals specify:

KIND_____

SEASON_____

DURATION IN MONTHS_____

SOIL PH

Q-59) Have you ever applied superphosphate or other phosphate fertilizer to the grazing land the last five years?

1. YES
2. NO

Q-60) if you answered “yes” above fill in:

BRAND NAME_____

SEASON_____

WHEN WAS THE LAST YEAR_____

Q-61) Was the fertilizer fortified with molybdenum?

1. YES
2. NO

Q-62) Have you ever applied limestone to disinfect the paddock the last five years?

1. YES
2. NO

Q-63) if answered “yes” above fill in:

SEASON_____

REPETITIONS PER YEAR_____

WHEN WAS THE LAST YEAR_____

MANURE MANAGEMENT

Q-64) How often do you clean the manure?

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1. NEVER (1-2 YEAR)
2. SELDOM (EVERY MONTH)
3. FREQUENTLY(EVERY TEN DAYS)
4. ALWAYS(EVERY DAY)

Q-65)Where do you dispose off the manure?

1. PASTURE
2. TO THE FIELDS THAT ARE CULTURED FOR PASTURE
3. ONE AND TWO
4. DOWN SLOPE
5. OTHER_____

Q-66) If you spread the manure to the field cultured for pasture, specify:

HOW MANY TIMES PER YEAR_____

IN WHICH MONTHS_____

INDUCTION OF STRESS DURING ROUTINE MANAGMENT

Q-67) When do you shear the does?

MONTH_____

Q-68) When do you ear-mark the does?

MONTH_____

Q-69) When do you dehorn the does?

MONTH_____

Q-70) When do you castrate the male kids?

MONTH_____

Q-71) Are there any other diseases in the flock currently diagnosed or under treatment?

1. MAMMARY
   GLAND_____
   _____
   _____
   _____
2. RESPIRATORY
   SYSTEM_____
   _____
   _____
   _____
3. GASTROINTESTINAL
   TRACT_____
   _____
   _____
   _____

Q-72) Score the nutritional condition of the does

1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐