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First report of *Besnoitia besnoiti* seropositive cattle in Greece

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Πρώτη αναφορά οροθετικότητας βοοειδών σε *Besnoitia besnoiti* στην Ελλάδα

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ABSTRACT. Aim of this study was to investigate presence of *Besnoitia besnoiti* infection in indigenous and imported cattle in Greece. A total of 450 bovine blood sera samples from 15 dairy herds of Northern Greece were tested using a commercially available kit to detect specific anti-*B. besnoiti* antibodies. Ninety-eight cows were found to be seropositive to *B. besnoiti* (22%). Seropositivity varied among herds from 0% to 35%. Results were analysed using a univariate analysis of variance. A full factorial model was developed, using as independent variables area of sampling (Thrace, Macedonia) and origin of cattle (local, imported). No significant effect was found of the variables included into the model ($P > 0.05$). It is concluded that seropositive dairy cows are present in Northern Greece; however, impact of this parasite on animal production in Greece remains a key topic for further investigation.

Keywords: *Besnoitia besnoiti*, dairy cattle, emerging disease, Greece, protozoan parasite

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ΠΕΡΙΛΗΨΗ. Η προσβολή των βοοειδών από το πρωτόζωο παράσιτο *Besnoitia besnoiti* προκαλεί νόσο ιδιαίτερης σημασίας, λόγω της έντασης των συμπτωμάτων που εκδηλώνονται κυρίως με πάχυνση, οιδήματα και νεκρώσεις του δέρματος. Η κλινική εκδήλωση της νόσου συνοδεύεται από μείωση της παραγωγής, με απώλεια σωματικού βάρους, μειωμένη γονιμότητα των ταύρων και, σε αρκετές περιπτώσεις, το θάνατο λόγω καχεξίας. Ο τρόπος μετάδοσης του παρασίτου δεν είναι εντελώς γνωστός μέχρι σήμερα, αν και πιθανότατα μεταφέρεται μηχανικά με αιματοφάγα έντομα. Η εξάπλωσή του από τις περιοχές της Ευρώπης όπου η νόσος ενδημεί γίνεται κυρίως με αγοραπωλησίες και μεταφορές ζώων. Το νόσημα βρέθηκε σε πολλές ευρωπαϊκές χώρες, ιδιαίτερα στις μεσογειακές, όπως η Ισπανία, η Ιταλία και η Πορτογαλία. Εντούτοις, δεν υπάρχει καμία σχετική πληροφορία για την ύπαρξη του παρασίτου στην Ελλάδα. Για το σκοπό αυτό, εξετάστηκαν 450 οροί αίματος γαλακτοπαραγωγών αγελάδων από 15 εκτροφές στη Μακεδονία και τη Θράκη, χρησιμοποιώντας μία εμπορική διαγνωστική δοκιμή. Τα ζώα αυτά είχαν πρόσφατα εισαχθεί στη χώρα μας από το εξωτερικό ή αποτελούσαν ζώα που γεννήθηκαν στις εκτροφές. Ανιχνεύθηκαν ειδικά αντισώματα συνολικά σε 98 (22%) ζώα. Η διακύμανση της συχνότητας των ζώων με ειδικά αντισώματα στις εκτροφές που εξετάστηκαν κυμαινόταν από 0% έως 35%. Η επεξεργασία των αποτελεσμάτων έγινε με τη χρήση μοντέλου που περιελάμβανε ως μεταβλητές την περιοχή και την προέλευση των ζώων. Δεν παρατηρήθηκαν σημαντικές διαφορές μεταξύ των ζώων στη Μακεδονία ή στη Θράκη, ούτε μεταξύ ζώων εκτρεφόμενων στην Ελλάδα ή εισαγωγής. Αυτή είναι η πρώτη αναφορά οροθετικών βοοειδών έναντι της *B. besnoiti* στη χώρα μας. Η έγκαιρη ανεύρεση των μολυσμένων ζώων είναι σημαντική για την αποφυγή της επέκτασης του νοσήματος. Τέλος, είναι αναγκαία η διερεύνηση της συχότητας της νόσου στα διαφορετικά συστήματα εκτροφής βοοειδών στην Ελλάδα.

Λέξεις ευρετηρίασης: *Besnoitia besnoiti*, αναδυόμενο νόσημα, γαλακτοπαραγωγή βοοειδή, Ελλάδα, πρωτόζωο παράσιτο

INTRODUCTION

Besnoitia besnoiti is a cyst-forming protozoan parasite of cattle, the causative agent of bovine besnoitiosis, which is a chronic and debilitating vector-borne disease. The disease is characterised by local and systemic clinical signs of varying severity, resulting to severe economic losses due to mortality, weight loss, prolonged convalescence, definitive or transient sterility in males, decline in milk production and potential abortion in dams and reduced value of the hides of the affected animals (Cortes et al., 2005; Jacquet et al., 2010; Alvarez-Garcia et al., 2013). The most characteristic clinical signs of chronic besnoitiosis are visible tissue cysts in the scleral conjunctiva and the vagina, thickened skin and a generally poor body condition (Frey et al., 2013).

Besnoitiosis, though it occurs endemically in many countries of Africa and Asia, in Europe until recently it was considered as a neglected disease restricted only to certain areas of Southern Europe, mainly Portugal, South France and Spain. However, during the last years, a spread of this disease has been reported to other parts of France and to previously *B. besnoiti*-free countries, including Germany, Italy and Switzerland (Cortes et al., 2005; Schares et al., 2009; Jacquet et al., 2010; Gentile et al., 2012; Alvarez-Garcia et al., 2013; Basso et al., 2013).

Aim of this study was to investigate the potential exposure of cattle in Greece to this parasite.

MATERIALS AND METHODS

Animals and blood sera samples

A total of 450 bovine blood sera samples were used in this study. Sampled animals were dairy cows (>2 years old) from 15 farms located in Northern Greece, 11 of these in Macedonia (n=330 animals) and 4 in Thrace (n=120 animals). The animals were characterised as either 'local' cows, i.e. born or having lived in Greece for over two years (n=184 from Macedonia and n=54 from Thrace) or 'imported' into the country, i.e. ones that have been imported into the country less than two years ago (n=146 from Macedonia and n=66 from Thrace). Imported animals were originating from France, Germany or the Netherlands. Blood sampling collection took place from April to November 2010.

Blood samples (5 mL) were collected from each animal individually into sterile glass vials with no anticoagulant. Vials were left in a standing position for 20 to 30 minutes until blood clotted and then were centrifuged at 1500 rpm for 10 min; serum was removed into plastic tubes and stored at -20 °C.

Detection methods

PrioCHECK® Besnoitia Ab (Prionics AG, The Netherlands), a commercially available kit was used according to manufacturers instructions to detect specific anti-*B. besnoiti* antibodies in the serum samples. This assay was based on ELISA technology, using plates coated with *Besnoitia* antigen and followed a four step protocol. The Optical Density (OD) of the wells was measured in a plate reader at 450 nm using a reference filter at 620 nm. Results of the assay were calculated according to the below formula:

$$\text{OD}_{450} \text{ of test sample} / \text{OD}_{450} \text{ of positive control} \times 100 = \% \text{ positivity.}$$

A percent positivity >20% was regarded as indicative of *B. besnoiti* infection.

Statistical analysis

Univariate analysis of variance was performed (IBM SPSS Statistics v. 21). A full factorial model was developed using as independent variables the area of sampling (Thrace, Macedonia) and the cows origin (imported, local). These variables were introduced into the model as fixed effects, whereas the model also accounted for their interaction term. Level of significance was set at $P < 0.05$.

RESULTS

In total, 98 (22%) cows from 15 herds were found to be seropositive to *B. besnoiti*. Seropositivity in herds varied from 0% to 35% (Table 1). Also, in total 48 (24%) seropositive cows were considered as 'imported' and 50 (20%) as 'locals' ($P = 0.449$). No significant effect was found of the variables (area and origin of the sampled cows), introduced into the model ($P > 0.05$). Detailed results are in Table 1.

DISCUSSION

During the last years, significant spread of bovine besnoitiosis is being observed, accounting for many outbreaks, also in regions traditionally free of this disease. In Portugal, Spain, France and, recently, Italy the disease is considered to be endemic (Basso et al., 2011; Alvarez-Garcia et al., 2013). Herein is reported for the first time, the detection of seropositive cows for *B. besnoiti* also in Greece. This finding confirms the

statement that besnoitiosis is a re-emerging disease in Europe, particularly in the Mediterranean basin.

Although trade of animals from endemic European regions was possibly involved in spread of the disease, there are still many aspects of the epidemiology of bovine besnoitiosis that are poorly understood. It has been assumed that this parasite, like other cyst-forming coccidia, has an indirect life cycle with cattle (and wild ruminants, i.e. antelopes) as intermediate hosts being infected with oocysts shed in the faeces of the definitive host. The theory that this is a carnivore animals has not yet been confirmed, since all attempts have failed to confirm this hypothesis, including other animal species as well as canids, reptiles, birds and many others ((Jacquet et al., 2010; Basso et al., 2011; Gutierrez-Exposito et al., 2013).

Currently, the only known mode of transmission for *B. besnoiti* is mechanical transmission, i.e. tachyzoites or bradyzoites inoculated intravenously or subcutaneously. This mode of transmission may possibly occur iatrogenically, with repeated usage of needles, or via blood-sucking insects, particularly tabanid species and stable flies, which represent the most important natural mode. However, in experiments carried out by Bigalke (1968), it has been shown that *B. besnoiti* was able to survive only for a short time in the above insects (<24 h in tabanids and 1 h in stable flies, *Stomoxys calcitrans*), making impossible a transmission over large distances by insects. The same researcher (Bigalke, 1968) also demonstrated experimentally that cyst stages (bradyzoites) inoculated into nostrils were able to cross mucous membranes and become infectious to cattle. This may also suggest that mucous membranes may rupture during mating representing another way for transmission by direct contact.

In Greece, climatic conditions allow survival of protozoa for long periods and development of high density populations of these blood-sucking insect species capable of transmitting *B. besnoiti*. Therefore, risk of maintaining and spreading this disease in the cattle population, including wild ruminants, of the country is significant. Particularly with animal trade and movements, if infected cattle enter a naive herd, it is possible that insects will facilitate spread of the infection within the herd (Cortes et al., 2005; Alvarez-Garcia et al., 2013). In our study, seropositive cattle had been im-

ported into Greece, highlighting this hypothesis as an important threat to the Greek livestock industry.

Chronic besnoitiosis characterized by dermal lesions is associated with the presence of macroscopic tissue cysts and is easily diagnosed, contrary to the acute febrile phase, which is characterized by anasarca and necrosis of skin, associated with multiplication of tachyzoites in vascular endothelium; this phase is short-lived and rarely diagnosed (Dubey et al., 2013). Serological identification of subclinically infected cattle is important to avoid introduction of infected animals into naive herds. Schares et al. (2010) have evaluated the sensitivity and specificity of the *Besnoitia*-Ab serological test PrioCHECK®, the test which was also used in our study. They concluded that it was a valuable diagnostic tool to detect infected animals and did not show cross-reactions with *Toxoplasma gondii* and *Neospora caninum* infections. Thus, it may be used to support control measures that include the separation of infected animals from the remaining herd to avoid a further transmission of the infection within the herd (Schares et al., 2010). Furthermore, the same authors concluded that the repeat-

ed transmission of *B. besnoiti* stages mechanically via blood sucking insects might impact level of antibodies against the parasite. If sampling occurs during winter months, when blood insect activity is low, cattle are not boosted to a sufficient extent with parasite antigens to maintain a detectable level of antibodies. In animals with a parasite load sufficient to cause tissue cysts detectable by clinical examination, the antigen load may have been sufficient also to maintain high antibody levels until the next spring. In another study, it was demonstrated that new *B. besnoitia* seroconversions occurred throughout the year with the highest number in spring. However, in the same study it was noted that seroconversion took also place in the two months before turn-out and could be associated with a high indoors activity of *S. calcitrans* during this period (Lienard et al., 2011). Blood sampling in our study took place during spring and autumn, during a period with high insect activity, and therefore we were able to detect most individuals challenged with the protozoan agent.

Overall prevalence of the positive animals was 22%. This prevalence is lower than the one reported

Table 1. Number and frequency (%) of seropositive for *Besnoitia besnoiti* cattle, imported or local, in farms in Northern Greece.

Area	Number of tested samples per farm	Imported (%)	Local (%)	Total (%)	P -value
Thrace	30	8 (53)	0 (0)	8 (27)	0.510
	45	2 (10)	4 (16)	6 (13)	
	30	0 (0)	2 (10)	2 (7)	
	15	0 (0)	0 (0)	0 (0)	
Macedonia	43	3 (23)	12 (40)	15 (35)	0.069
	35	8 (40)	4 (27)	12 (34)	
	30	4 (29)	6 (38)	10 (33)	
	35	6 (30)	4 (27)	10 (29)	
	37	8 (40)	2 (12)	10 (27)	
	50	3 (15)	9 (30)	12 (24)	
	30	1 (10)	5 (25)	6 (20)	
	40	4 (27)	2 (8)	6 (15)	
	10	1 (20)	0 (0)	1 (10)	
	10	0 (0)	0 (0)	0 (0)	
	10	0 (0)	0 (0)	0 (0)	
Total	450	48 (24)	50 (20)	98 (22)	0.449

by Rinaldi et al. (2013) in Italy (a recently *Besnoitia*-endemic characterised country), where farm prevalence was 83% and individual animal prevalence 44% in a study of 528 serum samples from cattle examined with an enzyme-linked immunosorbent assay test. In our case, mean seropositivity varied among herds from 0% to 35% (Table 1).

It is worth noting that many seropositive animals remain subclinically infected with no visible signs of disease, but according to Frey et al. (2013) they may still be able to transmit the parasite. During an outbreak of bovine besnoitiosis in Spain, most of the animals were seropositive (91%), but only 43% of seropositive cattle developed clinical signs compatible with besnoitiosis. Finally, a significant increase in seroprevalence and clinical signs was found to be associated with increasing age of the animals, suggesting rapid horizontal transmission of the disease (Fernandez-Garcia et al., 2010).

CONCLUDING REMARKS

Introduction of subclinically infected cattle into

naive herds seems to play a major role in transmission of the infection between herds, as well as between countries. Therefore, use of sensitive and specific serological tests is necessary to detect infected cattle in order for preventing introduction into non-infected herds. Seropositive cows are present in Northern Greece at 22%, equally distributed within recently imported and locally bred herds. Impact of the disease still remains unknown in Greece and needs further investigation. Further investigation is needed to demonstrate additional risk factors present in infected herds of Northern Greece that may facilitate spread of the parasite. Also, impact of besnoitiosis on Greek dairy cattle production needs to be evaluated. This may raise public concern to take immediate prevention steps as control or treatment tools of this disease do not exist yet.

CONFLICT OF INTEREST STATEMENT

None of the authors of this article has any conflict of interest. ■

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