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PII: S1383-5769(19)30372-1

DOI: https://doi.org/10.1016/j.parint.2019.102021

Reference: PARINT 102021

To appear in: *Parasitology International*

Received date: 17 May 2019

Revised date: 1 November 2019

Accepted date: 5 November 2019

Please cite this article as: L. Villa, A.L. Gazzonis, C. Perlotti, et al., First report of Demodex bovis infestation in bovine besnoitiosis co-infected dairy cattle in Italy, *Parasitology International*(2019), https://doi.org/10.1016/j.parint.2019.102021

Short Communication

First report of *Demodex bovis* infestation in bovine besnoitiosis co-infected dairy cattle in Italy.

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Abstract

A form of generalized demodectic mange in two dairy cows infected with *Besnoitia besnoiti* is described. The herd was endemically infected with bovine besnoitiosis; an overall seroprevalence of *B. besnoiti* antibodies of 23.5%, that increased up to 43.5% considering only cows, was reported. Two out of the cows seropositive to *B. besnoiti*, at clinical examination presented skin nodules, widespread all over the body, and in particular in anterior regions. Skin biopsies from the region of the neck were collected and the nodules were microscopically examined through compression method. *B. besnoiti* tissue cysts were not revealed but a semi-solid yellowish content was evidenced with the presence of several mites, morphologically identified as *Demodex bovis*. Histological examination of skin biopsies evidenced slight acanthosis and hyperkeratosis of the epidermis and superficial dermatitis with oedema and macrophagic and eosinophilic infiltration. Cystic formations located in the deep dermis were lined by metaplastic squamous epithelium and severe cellular infiltration. A treatment with eprinomectin was attempted and clinical improvement of both cows was observed, particularly at the fifteenth day after treatment, with nodules reduced in size and mites in there degenerated. This is the first report of the co-infection of *D. bovis* infestation and bovine besnoitiosis in cattle. Furthermore, it was demonstrated that *D. bovis* circulates in the Italian cattle population, but subclinical forms could be underdiagnosed.

Keywords: *Demodex bovis*; *Besnoitia besnoiti*; Co-infection; Dairy cows; Histology; Eprinomectin.

Demodex bovis (Stiles, 1892) is a mite that lives in hair follicles and sebaceous glands. It causes bovine demodicosis or demodectic mange. The disease is characterized by follicular papules and nodules, especially in anterior body regions [1]. It can occur both in subclinical and in a generalized form. Transmission occurs through close contact between animals, and mainly from infested dams to offspring. Bovine demodicosis is a quite common disease in tropical areas, but it is rare in temperate regions [2]. Only scarce, not updated and scattered data are available concerning *D. bovis* in cattle in Italy. Mange is included among notifiable diseases according to Italian veterinary rules (D.P.R. 8 Febbraio 1954, n. 320, Titolo II, Capo XXVII – Rogna, Art. 146-149). Among others, nutritional deficiencies, physiological stress due to pregnancy and lactation, presence of other infections or infestations, are factors associated with episodes of bovine demodicosis. Concerning individual factors, the disease seems to be more prevalent in female young animals [3].

Bovine besnoitiosis, caused by the cyst-forming apicomplexan protozoa *Besnoitia besnoiti*, is a chronic and debilitating parasitic disease of cattle, characterized by both cutaneous and systemic manifestations, compromising animal welfare and responsible for economic losses on affected farms. In Europe, including Italy, bovine besnoitiosis is an emerging or re-emerging disease, with an increasing geographical distribution and the number of cases of infection [4]. Bovine besnoitiosis was previously reported both in dairy cows and in beef cattle in Northern Italy [5,6,7]. Furthermore, *Besnoitia* spp. specific antibodies were recently detected for the first time in Italy also in horses and donkeys reared in Northern regions [8]. However, the identity of *Besnoitia* species circulating in Italian equids is unknown to date.

A case of generalized bovine demodicosis in two *B. besnoiti* seropositive cows from a dairy farm in Northern Italy is reported.

The study was conducted in a dairy farm hosting 217 Holstein Friesian cattle located in the province of Brescia (Lombardy, Northern Italy), where clinical cases of bovine besnoitiosis were previously reported [9]. At that time, a serological screening for *B. besnoiti* was performed on the whole herd using the recommended approach of ELISA (ID Screen® Besnoitia Indirect 2.0, IDVET,

Montpellier, France) and confirmatory Western Blot. Western Blot was performed and interpreted according to Fernandez-Garcia et al. [10]. Briefly, a total of 4×10^7 *B. besnoiti* tachyzoites under non reducing condition were employed for electrophoresis. Tachyzoite antigens were transferred to nitrocellulose membranes and incubated with sera from cattle at a 1:20 dilution, followed by a peroxidase-conjugated anti-Bovine IgG antibody diluted at 1:1200 (Sigma-Aldrich, Saint Louis, USA). The presence of at least three bands in at least two of the three principal antigenic areas (area I: 72.5, 58.9 and 51.4 kDa; area II: 38.7, 31.8 and 28.5 kDa; area III: 23.6, 19.1, 17.4, 14.5 kDa) was considered as a positive result. A seroprevalence of *B. besnoiti* of 23.5% was recorded, which increased up to 43.5% considering only adult cows [9]. The farm is officially free of tuberculosis, brucellosis and leucosis. All the animals of the herd are vaccinated against BVDV; besides, the farm adheres to the plan for the control of IBR and paratuberculosis. Further, some cases of Q Fever were diagnosed in the herd one year before and after those episodes all the animals were vaccinated against *Coxiella burnetii*.

During the clinical examination of the cows, which resulted seropositive to *B. besnoiti*, skin biopsies from the region of the neck were collected from three out of seven cows presenting skin lesions suggestive of bovine besnoitiosis. Skin biopsies were collected using 4- to 6-mm disposable punch instruments. An aliquot of the nodules was microscopically examined through compression between glasses to detect the presence of *B. besnoiti* tissue cysts. The material included in the nodules was dissolved in 5% potassium hydroxide (KOH) solution at room temperature for 2 hours to clear the mites. Subsequently, the material was filtered and washed from the colloidal remains and observed under a light microscope [11]. Mites were identified according to morphological characteristics as described by Bukva [12]. Another part was processed for histological examination: samples were fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 5µm, stained with hematoxylin and eosin (HE) and microscopically examined.

Two three years old lactating Holstein Friesian cows resulted seropositive for *B. besnoiti*: ELISA S/P% was 132 and 162, respectively, and Western Blot positive for both cows [9]. These animals

were clinically examined to reveal signs and lesions ascribable to bovine besnoitiosis. Any clinical alteration of the acute phase of the disease was not detected. Regarding the presence of *B. besnoiti* tissue cysts in typical localizations, i.e. skin, sclera and vulva, one of these cows presented tissue cysts in *scleral conjunctiva* and *vestibulum vaginae*. Besides, both cows evidenced the presence of skin nodules of varying sizes (0.5-2 cm), widespread all over the body, in particular in the regions of head, neck, back and flanks (Fig. 1.A). However, these skin lesions did not show the typical aspect of those characteristic of chronic bovine besnoitiosis. Indeed, the compression between glasses of the nodules did not reveal the presence of *B. besnoiti* tissue cysts, whereas a semi-solid yellowish content was found. The microscopic observation of this material after washing with 5% KOH evidenced the presence of mites, morphologically identified as *D. bovis* [12]. A huge quantity of mites at various stages of development was observed (Fig. 1.B). Histology showed slight acanthosis and hyperkeratosis of the epidermis and superficial dermatitis with oedema and macrophagic and eosinophilic infiltration. Cystic formations located in the deep dermis were lined by a layer of metaplastic squamous epithelium and severe infiltration of macrophages, plasma cells, eosinophils and lymphocytes (Fig. 2.A). The material included consisted of degenerated and necrotic granulocytes with parts of hair, keratin flakes and sectioned mites (Fig. 2.B). Any *B. besnoiti* tissue cyst was not detected at histological examination.

In these animals a treatment with eprinomectin (EPRINEX Pour-On 0.5%) was attempted [13]. This treatment was chosen since there is no milk withdrawal period for lactating dairy cattle and then milk from cows may be used for human consumption at any time following treatment. Two weeks later, these cows evidenced a clinical improvement: nodules were reduced in size and mites in there degenerated (Fig. 1.C). Another dose of treatment was administered one month after the first one, resulting in a further improvement of the clinical signs.

A generalized form of bovine demodectic mange was confirmed in two *B. besnoiti* co-infected cows from an endemically infected dairy cattle herd in Northern Italy. In the study farm, a high intra-herd seroprevalence of *B. besnoiti* antibodies and the presence of clinical signs of bovine besnoitiosis in

a part of the seropositive animals were previously reported [9]. Furthermore, this study highlighted the infestation with *D. bovis* in two cows positive to *B. besnoiti*. Unfortunately, it was not possible to understand how long the animals have been affected by *B. besnoiti* and no relevant clinical sign was reported in the past. The presence of lesions ascribable to bovine demodicosis was not observed in any other animal of the herd at the clinical examination; however, subclinical forms of the disease could not be excluded, since the presence of *D. bovis* was not systematically investigated in all cattle in the farm.

Only scarce, not updated and scattered data are available concerning *D. bovis* in cattle in Italy, where cases of bovine demodicosis were reported in cattle farms in Northern and Central Italy [14,15,16,17,18].

In temperate climatic zones, bovine demodicosis generally occurs in a subclinical chronic form with only a moderate nodule number; on the contrary, a generalized form with hundreds to thousands of nodules was reported in 2-5% of cattle under intensive condition in Europe [1].

The distribution of the nodules on the body surface of parasitized cows reflected what previously reported by Matthes [1], i.e. a predilection for anterior body regions (shoulders, arms, neck, dewlap and hypochondriac region). However, in these cows, many nodules were also detected in the cephalic region, as previously reported in three cows by Giammarino [18]. On the other side, only a few nodules were evidenced in back regions in both animals.

Bovine demodicosis is reported mainly in dairy cows with increased stress: the occurrence of the disease seems to be correlated to debilitating factors or to receptive physiological states of the animal (pregnancy or lactation). In this regard, it is important to underline that both cows were lactating. In particular, one cow was at the end of lactation (290 days), but still not pregnant, and was producing 33.9 kg of milk per day; instead, the other one was at the beginning of lactation (65 days) with a milk daily production of 46.6 kg. Furthermore, even this is not the case, immunosuppression during the peripartum period may also facilitate *D. bovis* infestation and skin colonization. Besides, the eventual interference between *B. besnoiti* and bovine demodicosis and/or

other infectious diseases should be clarified. In fact, since bovine besnoitiosis is a chronic and debilitating disease [19], it should not be neglected its role in determining an immune imbalance possibly leading to other infections, including *D. bovis*. Concerning *D. bovis*, both natural and acquired immunity contribute to reducing mite numbers and associated clinical signs in infested cattle. However, generalized demodicosis could be due to diseases altering the immune system. Indeed, the co-infection with bovine besnoitiosis may interfere with the immune response leading to severe form of the disease as described in the case of these two cows. It should also be considered that these cows were not only seropositive to *B. besnoiti*, but one of them was also clinically affected by the chronic form of bovine besnoitiosis, as demonstrated by the detection of tissue cysts in sclera and vulva. Concerning skin lesions, in these two cows, in all collected skin biopsies the presence of *B. besnoiti* was not evidenced both through compression between glasses and histology. However, it is not possible to determinate, only based on clinical signs, if any of the skin lesions may harbor also parasite tissue cysts, since visually it is not easy to distinguish between *B. besnoiti* tissue cysts and nodules due to *D. bovis* infestation. Moreover, Abu-Samra et al. [20] investigated the association between *Demodex* mites and bacteria involved in skin lesions of bovine demodicosis. Also in this case, the nature of the exudate suggested the involvement of bacteria, even if isolation of skin bacteria was not attempted.

This report demonstrated that *D. bovis* does circulate in the Italian cattle population. The disease should be further investigated. However, since bovine demodicosis is more frequently only subclinical, clinical forms could be underdiagnosed. Even if the disease does not compromise the life of infested animals, it could reduce the productivity of the herd. The treatment with Eprinomectin 0.5% Pour on lead to an improvement of the clinical signs in both cows; however, a mite-free status of the animals cannot be guaranteed.

The co-infection of *B. besnoiti* and *D. bovis* was confirmed in two dairy cows. This is the first detection of demodectic mange in bovine besnoitiosis infected cattle. Both diseases in cattle should be further investigated, also in relation to the immune response of infected animals.

Ethical statement

Biological samples were collected by qualified veterinarians applying adequate procedures of handling and disinfection to minimize pain or distress in sampled animals. All procedures for the collection of biological specimens from live animals were accomplished following good clinical practices in the respect of animal welfare according to current legislation. The study was conducted with the approval of the Institutional Animal Care and Use Committee of Università degli Studi di Milano (Permission OPBA_34_2017).

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest statement

The Authors declare they have no conflict of interest.

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Figure legends

Fig. 1. A. Head and neck of the infested cow with evidence of several skin nodules. B. Specimens of *Demodex bovis* at various stages of development before treatment. C. Degenerate mites after treatment with eprinomectin.

Fig. 2. Histopathology of skin nodules´ biopsies collected from *D. bovis* infested cows co-infected with *B. besnoiti*. A. Slight acanthosis and hyperkeratosis of the epidermis and superficial dermatitis. Cystic formations in the deep dermis lined by a layer of metaplastic squamous epithelium and severe cell infiltration (40X). B. Degenerated and necrotic granulocytes with parts of hair, keratin flakes and sectioned mites contained in cystic formations (400X).

Highlights

A case report of a generalized form of demodectic mange in two dairy cows in Italy

Demodex bovis infested cows were also seropositive to *Besnoiti besnoiti*

First report of the co-infection of *D. bovis* and bovine besnoitiosis

A treatment with Eprinomectin lead to an improvement of the clinical signs

D. bovis circulates in the Italian cattle but subclinical forms could be underdiagnosed

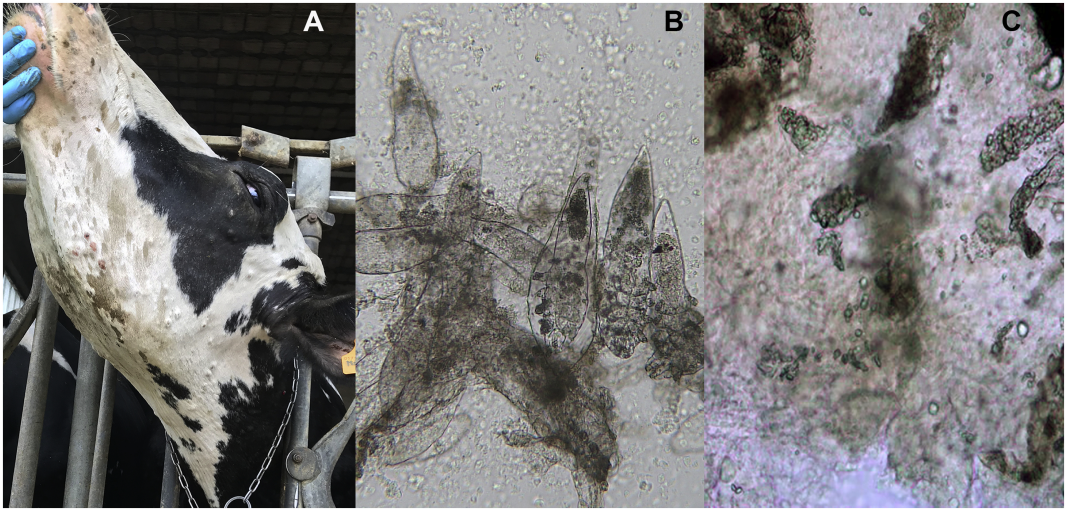

Figure 1

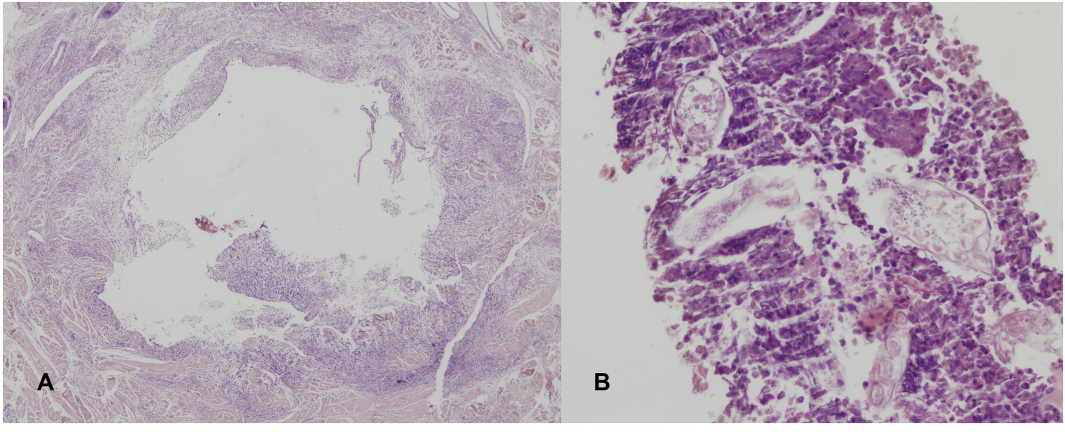

Figure 2