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Naturally acquired bovine besnoitiosis: Disease frequency, risk and outcome in an endemically infected beef herd

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Summary

The recent spread of bovine besnoitiosis warrants further epidemiological investigations to improve the knowledge on disease development. Thus, a 4-year longitudinal open cohort study was conducted in the first German cattle herd naturally infected with *Besnoitia besnoiti*. At seven herd-visits between 2008 and 2012, fourteen breeding bulls (>1.5 years) and 131 females (>1 year) were examined clinically and serologically. In females, clinical and serological prevalences, incidence and remission rates were determined. In addition, the association of age, antibody levels and number of visible parasitic cysts with clinical and serological outcome was investigated. The seroprevalence (89.4%–100%) and serological incidence rate (140.5 per 100 animal-years) were considerably higher than the clinical prevalence (23.5%–36.6%) and clinical incidence rate (16.7 per 100 animal-years). Of 33 new clinical and 12 new serological cases, only 6.7% (3/45) attracted attention with clinical signs of acute bovine besnoitiosis. The apparent serological remission rate (1.9 per 100 animal-years) was considerably lower than the clinical remission rate (37.3 per 100 animal-years). A median cyst score of <1 and mean immunofluorescent antibody test (IFAT) titre of ≤1,600 over the entire observation period was significantly associated with a negative clinical outcome at the end. Overall cyst score was not significantly associated with serological outcome and age had no significant influence on clinical and serological outcome. Within 4 years, there was a significant reduction in cyst scores and IFAT titres in the same animals, leading to eight clinically and serologically negative animals in the end. Two initially negative animals achieved clinical and apparent serological remission in about 2.5 years. In bulls, the time between herd entry and seroconversion was 7–30 months and the serological incidence rate was nearly identical to the rate in females (142.0 per 100 animal-years). This shows that a high *B. besnoiti* prevalence leads to infection of bulls within a short time period.

KEYWORDS
Besnoitia besnoiti, cohort study, cyst, incidence, prepuce, prevalence, remission, serological analysis

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1 | INTRODUCTION

Bovine besnoitiosis is an insidious disease of cattle caused by *Besnoitia besnoiti*, a cyst-forming apicomplexan parasite (Bigalke & Prozesky, 2004). In the past, the disease was indigenous in sub-Saharan Africa, Israel and in European regions with temperate climate: Southern France, Portugal and Spain (Álvarez-García, Frey, Ortega-Mora, & Schares, 2013). In recent years, however, bovine besnoitiosis expanded to Germany, Italy, Switzerland, Hungary, Belgium and Ireland (Basso et al., 2013; Gentile et al., 2012; Hornok, Fedák, Baska, Hofmann-Lehmann, & Basso, 2014; Rostaher, Mueller, Majzoub, Schares, & Gollnick, 2010; Ryan et al., 2016; Vanhoudt et al., 2015). This rapid increase in spread across national borders and geographical barriers is alarming and demands for a common European strategy for disease control (Álvarez-García et al., 2013; EFSA, 2010).

Acute bovine besnoitiosis is characterized by pyrexia, anorexia, peripheral lymphadenopathy, nasal and ocular discharge, lameness and subcutaneous oedema (Bigalke, 1968; Gollnick, Scharr, Schares, & Langenmayer, 2015). The lesions of this stage are induced by replicating tachyzoites within vascular endothelial cells (Basson, McCully, & Bigalke, 1970; Langenmayer, Gollnick, Scharr, et al., 2015). Severe chronic bovine besnoitiosis leads to thickening and wrinkling of the skin, alopecia and non-healing sole ulcers (Besnoit & Robin, 1912; Langenmayer, Gollnick, Majzoub-Altweck, et al., 2015). In bulls, infection can lead to orchitis, sterility and adverse effects on semen quality (Esteban-Gil et al., 2016; Kumi-Diaka, Wilson, Sanusi, Njoku, & Osori, 1981). In the chronic stage, typical parasitic cysts can be detected in various tissues. In some animals, large numbers of parasitic cysts are located in skin, scleral *conjunctivae* (SC), *vestibulum vaginae* (VV) and non-intestinal mucosa (Langenmayer, Gollnick, Majzoub-Altweck, et al., 2015; Majzoub et al., 2010; McCully, Basson, Van Niekerk, & Bigalke, 1966). In contrast to these peripheral tissues, visceral organs harbour markedly reduced parasite load, and the reasons for this remain unknown (Frey et al., 2013; Pols, 1960).

All breeds of cattle are considered intermediate hosts of the parasite, but despite several attempts to identify a potential definitive host, the presumed heteroxenous life cycle is still unknown (Basso, Schares, Gollnick, Rutten, & Deplazes, 2011; Diesing et al., 1988; EFSA, 2010).

Most infected animals display only mild clinical lesions, that is, pathognomonic SC or VV cysts, or are subclinically infected (Bigalke, 1968; Gutiérrez-Expósito, Ortega-Mora, et al., 2017; Jacquiet, Liénard, & Franc, 2010). Serological and PCR protocols have been established to detect *B. besnoiti*-specific antibodies and DNA to confirm clinical diagnoses and detect a majority of subclinical carriers (Cortes et al., 2006; Fernández-García et al., 2010; García-Lunar et al., 2013; Schares et al., 2010; Schares, Basso, et al., 2011; Schares et al., 2013).

The use of specific serological tests allowed large-scale herd examinations and comparative analyses of variations in seroprevalence with regard to different cattle management systems, age groups and gender, or the comparison of clinical prevalence and seroprevalence (Fernández-García et al., 2010; García-Lunar, Ortega-Mora, Schares, Diezma-Díaz, & Álvarez-García, 2017; Goldman & Pipano, 1983; Gutiérrez-Expósito, Ortega-Mora, et al., 2017; Liénard et al., 2011; Ryan et al., 2016). The different studies on clinical and serological prevalences have been recently reviewed (Gutiérrez-Expósito, Ferre, Ortega-Mora, & Álvarez-García, 2017).

However, long-term herd investigations in which state-of-the-art serological tests are routinely combined with clinical examinations are rare.

The objective of this study was to enhance the epidemiological understanding of bovine besnoitiosis including transmission rate and occurrence of the disease in an endemically *B. besnoiti*-affected herd. On the herd level, clinical and serological prevalences, incidence and remission rates were related to cyst numbers and antibody levels. On the individual animal level, disease course and outcome were investigated. Here, we report the results of a longitudinal open cohort study in which the same individual animals have repeatedly been examined clinically and serologically in seven independent herd checks over a period of 4 years, enabling coverage of the disease course on the herd level as well as the individual animal level.

2 | MATERIAL AND METHODS

2.1 | Abbreviations

The following abbreviations will be used throughout the manuscript: CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; HC, herd check; HE, haematoxylin and eosin; Herd-BbGer1, first cattle herd diagnosed with cases of bovine besnoitiosis in Germany; IFAT, immunofluorescent antibody test; OR, odds ratio; RR, relative risk; SC, scleral *conjunctivae*; *SD*, standard deviation; SP, specificity; VV, *vestibulum vaginae*.

2.2 | Herd history, management and animals

Herd-BbGer1, a southern Bavarian cow-calf-operation, was originally established in 1996 with animals from Hungary. Since 1999, a total of 55 cattle were purchased from France for restocking. Forty-four male and female animals originated from the French departments Cher, Indre and Saône-et-Loire, areas in which or close to which sporadic cases of bovine besnoitiosis had been reported at that time (Freudiger, 2008). Four additional cows purchased before 2004 came from the department Ariège (Region Midi-Pyrénées). In this department, bovine besnoitiosis had been reported as endemic since the 1950s (Dusart, 1956; Thomas, 2007). In August 2008, the first German case of bovine besnoitiosis was diagnosed. In 11/2008, only two cows born in France were still part of Herd-BbGer1. At the first HC (HC1) of this longitudinal open cohort study, Herd-BbGer1 consisted of 131 female Charolais and Limousin cattle older than 1 year, six breeding bulls and 77 male and female calves and young stock (Schares et al., 2010). In the following 4-year period, the herd was visited six more times and the remaining animals of the initial 131 were re-examined together with overall 14 Limousin and Charolais breeding bulls >1.5 years old (Female and male animal numbers in Table 1).

TABLE 1 Composition and number of clinical and serological examinations at respective herd checks (HCs) of Herd-BbGer1

Herd check designation (year, month)	HC1 (2008, 11)	HC2 (2009, 3)	HC3 (2009, 12)	HC4 (2010, 4)	HC5 (2010, 12)	HC6 (2011, 4)	HC7 (2012, 11)	Total number
Females >1 year	131	119	108	104	97	95	85	131
Breed females Charolais/Limousin	27/104	24/95	22/86	20/84	19/78	18/78	15/70	27/104
Average age of females tested (years)	5.5	5.9	6.4	6.7	7.1	7.5	8.8	na
Average age of 85 females followed until HC7 (years)	4.8	5.2	5.9	6.2	6.9	7.2	8.8	na
Breeding bulls >1.5 years	6	2	5	2	5	6	6	14
Breed of bulls, Charolais/Limousin	2/4	0/2	1/4	1/1	1/4	1/5	1/5	3/11
Number of examinations, female/male	131/6	119/2	108/5	104/2	97/5	95/6	85/6	739/32
Number of serological examinations, female/male	131/6	118/2	108/5	104/2	97/5	95/6	85/6	738/32
Number of clinical examinations, female/male	131/6	119/2	108/5	101/0	97/5	95/6	85/4	736/28

na, not applicable.

From November to April, animals were housed indoors in small groups. Cows were grouped by expected date of calving and by breed. During the grazing period, cows were kept in subherds based on their breed and the sex of their calf on seven different pastures up to 120 km apart. Breeding bulls were rotated between subherds of their respective breed. Animals were monitored daily by the owner. The only new animals entering the herd throughout the study period were eight breeding bulls purchased in France and Germany, which tested negative for *B. besnoiti* antibodies by an immunofluorescent antibody test (IFAT) and by two immunoblots (Schares et al., 2010).

2.3 | Clinical examination

Examinations and samplings were in accordance with national law and conducted by veterinarians during the specific herd health management routine in Herd-BbGer1. In 2008, the procedure was additionally supervised by the regional authorities. No ethical approval was required for this study.

Every clinical examination was performed by an experienced cattle veterinarian (NSG). Animals were restrained in a cattle chute, and the head was tethered. The examination premises for visual examination were illuminated with spotlights for direct and indirect lightening and headlamps. For each animal at each examination, the number of parasitic cysts in the mucous membrane of VV or prepuce and the mean number of cysts in the SC were estimated and assigned to the following scores: 1: 1–5 cysts; 2: 6–10 cysts; 3: 11–20 cysts; 4: 21–30 cysts; 5: >30 cysts. Animals with a cyst score of at least 1 in one location were defined as clinically positive.

2.4 | Blood sampling, serology and histology

Blood samples for serology were taken from the jugular or tail vein. An in-house enzyme-linked immunosorbent assay (ELISA) using affinity-purified *B. besnoiti* tachyzoite antigens was performed to examine all sera. In the ELISA, a cut-off of SP = 1.756 was applied (Schares et al., 2013). Sera with a SP value above this cut-off were defined as

positive. The serological status of every animal was defined by the ELISA result. To determine antibody levels, an IFAT was performed as previously described with a reciprocal titre of 200 as positive cut-off (Schares et al., 2010). In a few sera, serological results were confirmed by immunoblots using antigens of *B. besnoiti* tachyzoites and bradyzoites as previously described (Schares et al., 2010). Preputial samples of bull 215 were fixed, embedded, cut and HE stained as previously described (Langenmayer, Gollnick, Majzoub-Altweck, et al., 2015).

2.5 | Data analysis

Calculations and statistical tests were performed using Microsoft Excel (Microsoft Office 2016) and GraphPad Prism version 5.04 for Windows (GraphPad Software, La Jolla, California, USA).

Estimates for clinical and serological incidence rates per 100 animal-years were calculated based on the number of new cases per HC and the total of animal-years for follow-up. Animals negative at HC1 were included until their first positive test result. Animals lost to follow-up and animals becoming positive were assumed to be clinically or serologically negative half of the time period between HCs.

Likewise, clinical and apparent serological remission rates per 100 animal-years were estimated based on the number of new negative cases per HC and the total of animal-years for follow-up. Animals positive at HC1 were included until their first negative test result. Animals were regarded clinically negative, when the last clinical examination before follow-up loss was negative. Animals lost to follow-up and animals becoming negative were assumed to be clinically or serologically positive half of the time period between HCs.

Association measures of the exposure categories cyst score, IFAT titre and age on clinical and serological outcome and calculation of OR and RR were performed using two-by-two tables. For the analysis regarding clinical and serological outcome, every animal with a positive result at HC1 which was follow-up examined at least at five consecutive HCs was included (in total, 34 and 87 animals, respectively).

For exposure category cyst score, animals were grouped into two groups according to their median overall cyst score, which was calculated for every individual animal from every clinical examination. One group comprised animals with a median overall cyst score <1 and the other animals with a median overall cyst score ≥1. For exposure category IFAT titre, animals were grouped into two groups according to their mean IFAT titre calculated for every individual animal from every serological examination. One group comprised animals with a mean reciprocal IFAT titre ≤1,600 and the other animals with a mean reciprocal IFAT titre >1,600. For exposure category age, animals were grouped into three groups (<2; 2–5; >5 years) according to their age at HC1.

3 | RESULTS

3.1 | Clinical and serological findings and prevalence

In total, 771 examinations were performed, $n = 739$ in female and $n = 32$ in male animals (Table 1). In 764 examinations, both serological and clinical results (results of at least one examination site: SC, VV or prepuce) were obtained (in females: $n = 736$; in males: $n = 28$). In 235 clinical examinations (in females: $n = 229$; in males: $n = 6$), cysts as signs for chronic bovine besnoitiosis were documented at the examination sites (SC, VV or prepuce). Visible tissue cysts were observed only in the VV in 47/229 (20.5%) of clinical examinations, only in the SC in 67/229 (29.3%) of clinical examinations and cysts in both locations in 115/229 (50.2%) of clinical examinations. Skin lesions consistent with bovine besnoitiosis were observed in 14/131 (10.7%) females. Only in 1.7% (4/235) of the exams, few SC or VV cysts were documented in females, which tested seronegative at the respective HC. The four sera of these

females were also tested with tachyzoite and bradyzoite antigen immunoblots, which revealed also negative results.

During the 4-year period, 46 animals were removed from the herd, leaving 85 of the initial 131 female cattle at HC7. Table 2 summarizes clinical and serological findings in all females and in the group of 85 females which had been examined at each HC. Seroprevalence was high at all HCs (89.4%–100%). The seroprevalence increased from 90% to 100% during the first 2 years and dropped to 89.4% at HC7. The differences between seroprevalences of all HCs in all females and the group of 85 were highly significant ($p < .001$, chi-square test). Clinical prevalences were considerably lower with highest values at HC1 (36.6%) and lowest value in HC7 (23.5%). The differences between clinical prevalences of all HCs in all females and the group of 85 were not significant ($p > .05$, chi-square test). The majority of clinically positive animals displayed mild clinical signs, that is, few SC or mucosal cysts.

3.2 | Clinical and serological incidence and remission rates

Of 83 clinically negative female cattle at HC1, 12 were lost to follow-up and 33/71 (46.5%) acquired SC or VV cysts until HC7 (Table 3). Animals were observed for 2,366 months, which equals 197.1 animal-years and results in an incidence rate of 16.7 per 100 animal-years. In total, 21/33 (63.6%) new chronic clinical cases occurred before HC3. Of 30 new chronic clinical cases which were observed before HC7, 17 appeared in the time period between autumn and spring and were first detected at the spring HCs, resulting in an autumn:spring ratio of 1:1.3.

Of 48 clinically positive female cattle at HC1, 14 were lost to follow-up and 29/34 (85.3%) lost SC or VV cysts until HC7. Animals were observed for 934 months, which equals 77.8 animal-years, resulting in a remission rate of 37.3 per 100 animal-years. Of 24 new negative clinical cases which were observed before HC7, 20

TABLE 2 Serological and clinical prevalence of *Besnoitia besnoiti* infection at each HC regarding the group of all present female cattle older than 1 year, and the group of 85 cattle which were tested repeatedly over the 4-year period

Group/Parameter	Herd check designation (year, month)						
	HC1 (2008, 11)	HC2 (2009, 3)	HC3 (2009, 12)	HC4 (2010, 4)	HC5 (2010, 12)	HC6 (2011, 4)	HC7 (2012, 11)
Animals >1 year	131	119	108	104	97	96	85
Seroprevalence	90.1% (118/131)	92.4% (110/119)	98.1% (106/108)	99.0% (103/104)	100% (97/97)	100% (96/96)	89.4% (76/85)
Proportion: C+/S+	38.1% (45/118)	36.4% (40/110)	27.4% (29/106)	35.0% (36/103)	35.1% (34/97)	26.0% (25/96)	25.0% (19/76)
Clinical Prevalence	36.6% (48/131)	33.6% (40/119)	26.9% (29/108)	34.6% (36/104)	35.1% (34/97)	26.0% (25/96)	23.5% (20/85)
Proportion: S+/C+	93.8% (45/48)	100% (40/40)	100% (29/29)	100% (36/36)	100% (34/34)	100% (25/25)	95.0% (19/20)
Animals present at all HCs	85	85	85	85	85	85	85
Seroprevalence	90.6% (77/85)	94.1% (80/85)	97.6% (83/85)	98.8% (84/85)	100% (85/85)	100% (85/85)	89.4% (76/85)
Proportion: C+/S+	33.8% (26/77)	37.5% (30/80)	26.5% (22/83)	32.1% (27/84)	31.8% (27/85)	25.9% (22/85)	25.0% (19/76)
Clinical Prevalence	31.8% (27/85)	35.3% (30/85)	25.9% (22/85)	31.8% (27/85)	31.8% (27/85)	25.9% (22/85)	23.5% (20/85)
Proportion: S+/C+	96.3% (26/27)	100% (30/30)	100% (22/22)	100% (27/27)	100% (27/27)	100% (22/22)	95.0% (19/20)

C+, clinical status positive; S+, serological status positive.

TABLE 3 Clinical and serological incidence and remission of bovine *Besnoitia besnoiti* infections in Herd-BbGer1

Herd check designation (moo)	Cohort of 83 cyst negative animals at HC1, clinical incidence			Cohort of 48 cyst positive animals at HC1, clinical remission			Cohort of 13 seronegative animals at HC1, serological incidence			Cohort of 118 seropositive animals at HC1, serological remission		
	Remaining negative animals (lost to follow-up)	New cyst positive animals	%	Remaining positive animals (lost to follow-up)	New cyst negative animals	%	Remaining negative animals (lost to follow-up)	New seropositive animals	%	Remaining positive animals (lost to follow-up)	New seronegative animals	%
HC2 (4)	68 (3)	12	15	29 (9)	10	25.6	9 (0)	4	30.8	106 (12)	0	0
HC3 (13)	53 (6)	9	14.5	26 (2)	1	3.7	2 (1)	6	75	96 (10)	0	0
HC4 (16)	48 (2)	3	5.9	24 (0)	2	7.7	1 (0)	1	50	93 (3)	0	0
HC5 (25)	43 (1)	4	8.5	22 (0)	2	8.3	0 (0)	1	100	88 (5)	0	0
HC6 (29)	41 (0)	2	4.7	13 (0)	9	40.9	0 (0)	0	0	87 (1)	0	0
HC7 (49)	38 (0)	3	7.3	5 (3)	5	50	0 (0)	0	0	67 (11)	9	11.8
Total	na	33	na	na	29	na	na	12	na	na	9	na

HC, herd check; moo, months of observation; na, not applicable.

appeared at the spring HCs, resulting in an autumn:spring ratio of 1:5.

Of 13 serologically negative female cattle at HC1, one was lost to follow-up and the remaining twelve animals (100%) seroconverted. Animals were observed for 103 months, which equals 8.5 animal-years, resulting in an incidence rate of 140.5 per 100 animal-years. The autumn:spring ratio was 1:1.4.

Of 118 serologically positive female cattle at HC1, 42 were lost to follow-up and 9/76 (11.8%) lost seropositivity, all of them before HC7. Animals were observed for 4,345 months, which equals 362.1 animal-years, resulting in an apparent remission rate of 1.9 per 100 animal-years. Eight of these nine cows were also clinically negative.

3.3 | Acute *B. besnoiti* infections in the group of female cattle

Of 33 new clinical and 12 new serological cases, only 6.7% (3/45) were observed by the owner with clinical signs consistent with acute bovine besnoitiosis (separation from the herd, nasal/ocular discharge). Two of these cases were included in a separate cohabitation trial (as Study animals 20 and 22), details of disease course were described elsewhere (Langenmayer, Scharr, Sauter-Louis, Schares, & Gollnick, 2015). The remaining animal separated from the herd in summer 2009 (between HC2 and HC3), showed nasal and ocular discharge and seroconverted shortly after clinical signs receded. At HC3, it was also clinically positive and was subsequently culled from the herd.

3.4 | Cyst scores are related to clinical outcome

To relate the clinical presentation at the beginning of the observation period with the outcome of infection after 4 years, we compared clinical and serological status of 85 animals at HC1 in 2008 and HC7 in 2012 (Table S1). To determine the association with clinical presentation, we calculated mean and median SC and VV cyst scores of all clinical examinations in all HCs of these animals.

At HC7, 20 animals were clinically positive and 65 animals clinically negative (Table S1). Cyst scores of every clinical examination with a clinically positive result of the 20 animals (HC1–HC7) were summarized for SC cysts (73 examinations, median = 3, mean = 2.63, SD = 1.56) and VV cysts (79 examinations, median = 3, mean = 2.75, SD = 1.55). Of the 65 animals, cyst scores of every clinical examination with a clinically positive result from HC1–HC7 were also summarized for SC cysts (62 examinations, median = 1, mean = 1.21, SD = 0.52) and VV cysts (49 examinations, median = 1, mean = 1.27, SD = 0.76). The difference in SC and VV cyst scores between clinically positive and clinically negative animals at HC7 was highly significant (Mann–Whitney test, $p < .0001$).

Of the 26 clinically and serologically positive animals at HC1 (Table S1, 1st row), the subgroup of seven animals (48 examinations) which stayed clinically and serologically positive had high SC (median = 3, mean = 2.81, SD = 1.65) and VV (median = 3, mean = 2.98, SD = 1.75) cyst scores in all examinations from HC1 to

HC7. The subgroup of 16 animals which became clinically negative but remained seropositive (111 clinical examinations) had low SC (median = 0, mean = 0.34, SD = 0.55) and VV (median = 0, mean = 0.25, SD = 0.45) cyst scores. The difference in SC and VV cyst scores between those two groups was also highly significant (Mann–Whitney test, $p < .0001$). To outline the clinical course from HC1 to HC7 of the two groups, the mean cyst scores are shown in Figure S1.

Of 51 clinically negative and serologically positive animals at HC1, 38 retained their status at HC7, four became clinically and seronegative in the end and nine developed SC or VV cyst at later HCs.

Of the group of 85, only seven animals were clinically and serologically negative at HC1 (Table S1, 4th row). Two of these developed signs of infection after HC2 displaying clinically and serologically positive results and achieved clinical and apparent serological remission until HC7 (Figure S2). Both animals had a maximum cyst score of three and high reciprocal IFAT titres (maximum of 6,400 and 1,600, respectively). Assuming clinical and serological negativity half of the time period between HCs, both animals achieved apparent remission in about 2.5 years.

Animals with a median overall cyst score <1 were significantly associated with a negative clinical outcome at HC6 or HC7 (Fisher's exact test, $p < .001$). The OR of positive clinical outcome at HC6 or HC7 with median overall cyst score <1 was 0.03, whereas the OR of negative clinical outcome at HC6 or HC7 with median overall cyst score <1 was 30. The risk of negative clinical outcome with a median overall cyst score <1 was 0.91, whereas the risk of negative clinical outcome with a median overall cyst score ≥1 was 0.25, resulting in a RR of 3.64 (95% CI = 1.35–9.78, Table S2).

The association between mean IFAT titre and clinical outcome at the last two HCs was significant (Fisher's exact test, $p < .01$). The OR of positive clinical outcome at HC6 or HC7 with mean IFAT titre ≤1,600 was 0.1, whereas the OR of negative clinical outcome at HC6 or HC7 was 9.6. The risk of negative clinical outcome with mean IFAT titre ≤1,600 was 0.86, whereas the risk of negative clinical outcome with mean IFAT titre >1,600 was 0.38, resulting in a RR of 2.23 (95% CI = 1.10–4.53, Table S3).

The association of the exposure category age with clinical or serological outcome and median cyst score and serological outcome was not significant (Fisher's exact test, $p > .05$).

3.5 | Reduction in cyst scores and IFAT titre over time resulted in few test negative animals in the end

To determine whether cyst numbers decreased over time, we compared cyst scores of 27 clinically positive animals at HC1 (Table 2) with the same animals' score at HC7. There was a highly significant reduction in SC cyst score from HC1 (median = 1, mean = 1.52, SD = 1.23) to HC7 (median = 0, mean = 0.56, SD = 1.2; Mann–Whitney test, $p < .0001$). There was also a significant reduction in VV cyst score from HC1 (median = 1, mean = 1.15, SD = 1.27) to HC7 (median = 0, mean = 0.7, SD = 1.43; Mann–Whitney test, $p < .01$).

Similarly, IFAT titres of 77 seropositive animals at HC1 (Table 2) were compared with the same animals' titres at HC7. There was a highly significant reduction in IFAT titre from HC1 (mean = 1,488, SD = 1,539) to HC7 (mean = 688, SD = 970; Mann–Whitney test, $p < .0001$).

Only eight female cattle were clinically and serologically negative at the end of the study (HC7). Only one of these was clinically negative at all HCs. Two of these were those animals which had developed first signs of bovine besnoitiosis during the study period and experienced apparent remission until HC7 (Section 3.4). All of these animals were additionally tested with immunoblots, which were also negative.

3.6 | Every breeding bull became infected with *B. besnoiti*

Table 4 shows the clinical and serological status of all 14 breeding bulls from HC1 until HC7. In 37.9% (11/29) of clinical examinations, the prepuce was examined. In only 18.2% (2/11) of preputial examinations, tissue cysts were diagnosed, both of them in bull 215. In 5/14 (35.7%) bulls, skin lesions consistent with bovine besnoitiosis were observed. Six bulls were in the herd at HC1, four of them were already infected with *B. besnoiti* and were subsequently culled from the herd: Three displayed few SC cysts, and one tested *B. besnoiti*-seropositive only. The remaining two negative bulls (Bulls 43 and 73) were introduced into the herd 15 and 4 months prior to HC1, respectively. Bull 43 seroconverted before HC3, but remained clinically negative from HC1 until HC7. Bull 73 seroconverted prior to HC2, but remained clinically negative until removed from the herd due to unsatisfactory reproductive performance. Eight newly purchased breeding bulls tested seronegative with IFAT and two immunoblots (Schares et al., 2010) prior to introduction into Herd-BbGer-1. All of them seroconverted during the study course. The time interval between herd entry and seroconversion was 7–30 months. Ten seronegative bulls were observed for 84.5 months (7.0 animal-years), resulting in an incidence rate of 142.0 per 100 animal-years. After introduction into the herd in April, the owner observed three bulls (Bulls 221, 222 and 215) with signs of acute bovine besnoitiosis in the following summer. These included separation from the herd, severe depression, anorexia and loss of body condition, nasal and ocular discharge, and lameness. Two of them developed SC cysts. Post-mortem examination of the reproductive organs of Bull 215 revealed several cysts in the mucosa of the penis and prepuce (Figure 1) and mild dermal parasitic DNA burden (Schares et al., 2016). The three bulls were culled from Herd-BbGer1 within 1 year.

4 | DISCUSSION

Epidemiological studies concerning bovine besnoitiosis are few, but the constant spread of the disease in Europe demands further epidemiological investigations in infected herds. The recent, well-documented history of bovine besnoitiosis in Herd-BbGer1 and the fact

TABLE 4 Clinical and serological status of all breeding bulls of Herd-BbGer1

Parameters					Herd check designation (year, month)						
#	Breed	Date of birth	Date of entry	Date of exit	HC1 (2008, 11)	HC2 (2009, 3)	HC3 (2009, 12)	HC4 (2010, 3)	HC5 (2010, 12)	HC6 (2011, 4)	HC7 (2012, 11)
70	Charolais	02/2007	06/2008	12/2008	C:+ S:+						
73[a]	Limousin	12/2005	08/2007	02/2010	C:− S:−	C:− S:+	C:− S:+				
80	Charolais	02/2004	02/2004	12/2008	C:− S:+						
169	Limousin	12/2005	06/2007	12/2008	C:+ S:+						
31	Limousin	07/2004	10/2005	11/2008	C:+ S:+						
43[a]	Limousin	03/2004	07/2008		C:− S:−	C:− S:−	C:− S:+	C: na S:+	C:− S:+	C:− S:+	C:− S:+
221[a]	Limousin	05/2004	04/2009	02/2010			C:− S:+				
222[a]	Limousin	04/2004	04/2009	02/2010			C:+ S:+				
373[a]	Charolais	12/2002	04/2009				C:− S:+	C: na S:+	C− S:+	C:− S:+	C: na S: +
215[a]	Limousin	10/2002	04/2010	04/2011					C:+ S:+	C:+ S:+	
216[a]	Limousin	01/2005	05/2010						C:− S:−	C:− S:−	C:−S :+
218[a]	Limousin	01/2008	05/2010						C:− S:+	C:− S:+	C:− S:+
217[a]	Limousin	01/2007	04/2011							C:− S:−	C:− S:+
219[a]	Limousin	08/2008	12/2011								C: na S:+

C, clinical status; S, serological status; na, not applicable.
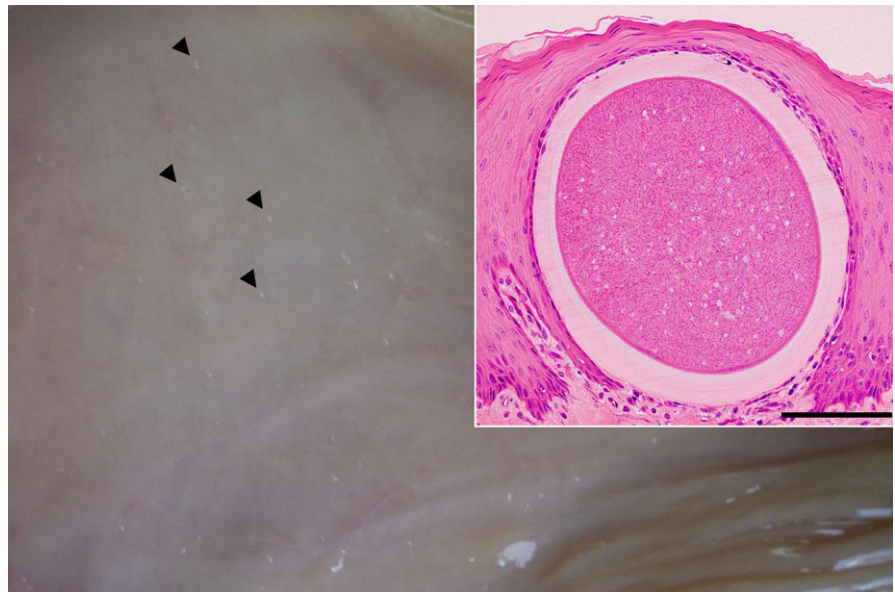[a]Bulls used to calculate serological incidence rate.

FIGURE 1 Prepuce of bull 215 with multifocal many miliary shiny tissue cysts (arrowheads). Inset: HE stained histological section of such a cyst. Note typical layering of *Besnoitia besnoiti* cyst with bulging and thinning of the preputial epithelium. Bar = 100 μm

that the disease is not widespread in Germany constitute an ideal scenario to study the dynamics of endemic disease in an isolated herd. A further strength of this study is the employment of a high number of breeding bulls in Herd-BbGer1 and the combination of sensitive serological tests and thorough clinical examinations yielding qualitative and quantitative data for individual animals. As clinical examinations were performed by only one experienced buiatrician and serological tests were conducted in one laboratory, the inter-observer and inter-laboratory variance is negligible, producing very comparable data.

The average seroprevalence in Herd-BbGer1 was 95% over the entire time of 4 years, and it was considerably higher than the average clinical prevalence, which was 27%. The average seroprevalence is considered very high, especially when compared with an another 4-year study, which reported an average seroprevalence of 50% (Gutiérrez-Expósito, Ortega-Mora, et al., 2017). However, the inter-herd prevalences may differ largely between herds and values above 90% are not unusual, especially in outbreak situations (Fernández-García et al., 2010; Gutiérrez-Expósito, Ortega-Mora, et al., 2017).

By contrast, the average clinical prevalence in Herd-BbGer1 was low in comparison with other studies, which reported an average clinical prevalence of 43% and 67% (Fernández-García et al., 2010; Gutiérrez-Expósito, Ortega-Mora, et al., 2017). Further studies reported clinical prevalences of 1%–10% in areas where bovine besnoitiosis is endemic (Jacquiet et al., 2010) and up to 22% in a longitudinal study of dairy cattle (Liénard et al., 2011). Differences between studies can originate from different definitions of clinical disease, inter-observer variability, different herd management and control measures (Gutiérrez-Expósito, Ortega-Mora, et al., 2017), potential differences in virulence between *B. besnoiti* strains, endemic condition of respective herds or a combination of several of these.

Clinical and serological incidence rates were quite high in this study. In a study on clinical and serological dynamics of bovine besnoitiosis over 4 years, a clinical incidence of up to 16.7 per 100 animal-years was reported in 26 animals studied (Gutiérrez-Expósito, Ortega-Mora, et al., 2017). In the present 4-year study on nearly three times as many animals ($n = 71$), the clinical incidence rate was the same. In contrast, the serological incidence in this study was considerably higher, that is, 140.5 per 100 animal-years compared with a maximum of 32 per 100 animal-years in the study by Gutiérrez-Expósito, Ortega-Mora, et al. (2017). This is most likely due to the very low number of seronegative animals at study start and the large proportion of infected animals. New clinical cases showed a tendency to occur between autumn and spring examinations, that is, were first detected at the spring HCs. This probably reflects the time interval from infection, which occurs preferably during summer, to appearance of first grossly visible tissue cysts. The incubation period of bovine besnoitiosis was reported as ~13 days (Basson et al., 1970; Bigalke, 1968). The time period from first day of fever until first appearance of SC cysts ranged between 37 and 49 days (Bigalke, 1968). In a previous study, we could confirm this time period; we observed first SC cysts in two naturally infected animals 28 and 38 days after beginning of acute stage (Langenmayer, Gollnick, Majzoub-Altweck, et al., 2015). This time frame of SC cyst development can explain the observed tendency, because animals infected in late summer need some time to display SC cysts and therefore appear first positive in the spring HCs.

The design of this study allowed the examination of the same individual cattle over a 4-year period, which enabled us to observe the disappearance of clinically visible parasitic cysts and to calculate a clinical and serological remission rate. Clinical recovery and negative seroconversion has been reported before (Gutiérrez-Expósito, Ortega-Mora, et al., 2017; Jacquiet et al., 2010), but remission rates of bovine besnoitiosis have not been determined so far. Interestingly, the clinical remission rate was quite high (37 per 100 animal-years) and significantly higher than the clinical incidence rate (16 per 100 animal-years) in this study, which eventually led to a reduction in clinical prevalence in the last two HCs. In contrast, the apparent serological remission rate was very low (~2 per 100 animal-years), emphasizing the long time needed to overcome the infection and to become serologically negative again. It is striking that the clinical remission rate is more than two times higher than the clinical incidence rate. The majority of animals achieved clinical remission in the time period between autumn and spring, that is, were negative the first time at the spring HCs. It is tempting to speculate that the observed seasonality might be associated with increased insect activity and parasite contact in summer and autumn.

Using association measures and comparisons between different subgroups, we were able to show that overall cyst scores (reflecting cyst numbers) were significantly associated with disease status and disease outcome. With a RR of 3.64, animals with lower cyst scores are three times as likely to result in a negative clinical outcome as animals with higher cyst scores. Likewise, with a RR of 2.23, animals with lower IFAT titre are two times as likely to result in a negative clinical outcome as animals with a higher IFAT titre. A correlation between parasite load and disease signs has also been reported previously (Frey et al., 2013; Gollnick et al., 2015).

Only four animals displayed SC and VV cysts but tested seronegative with the ELISA. Seronegative animals with clinical signs have been reported before in Herd-BbGer-1 and from other herds (Fernández-García et al., 2010; Gutiérrez-Expósito, Ortega-Mora, et al., 2017; Schares et al., 2010). As a confirmatory test, additional tests with immunoblots were included, which were also negative. Seronegative animals with detectable cysts pose a considerable threat to control programmes based on serology. To overcome this risk, a recent review recommends clinical and serological examination as diagnostic approach to control bovine besnoitiosis (Gutiérrez-Expósito, Ferre, et al., 2017).

Two animals achieved clinical and apparent serological remission in <2.5 years despite having relatively high SC and VV cyst scores. Obviously, these animals are exceptions of the model that low cyst scores favour negative clinical outcome. Because bradyzoites of *B. besnoiti* are intracellular, these animals probably developed a very strong *T*-cell response to eliminate the parasites and may represent "super-responders" and further studies are necessary to identify the true reasons for the highly efficient elimination of the parasite in those cattle.

Overall, there was a significant reduction in SC and VV cyst scores and mean reciprocal IFAT titre over the 4-year period. In 9.4% (8/85) of the animals, a clinical and apparent serological remission was observed. Fluctuations in antibody levels have been described (Gutiérrez-Expósito, Ortega-Mora, et al., 2017), leading to apparent serological remission of animals, which may be seropositive in future examinations. Whether or not these animals still harboured tissue cysts remains unknown, but if they do, these animals may propose a threat as disease spreaders because they are not identified by current tests. Future studies, looking on many different sampling sites per animal and optimally conducted with highly sensitive molecular techniques similar to a study to find differences between different skin locations (Schares et al., 2016) are necessary to study this aspect in those animals with an apparent complete remission.

In this study, 88.9% (8/9) of the animals which showed apparent serological remission were also clinically negative at HC7. Obviously, a negative result in the clinical examination is likely a prerequisite

for apparent negative seroconversion. This suggests that no or only very low parasitic loads are necessary for an animal to become serologically negative. Findings in previous studies are in accord with this: animals with high IFAT titres had a higher *B. besnoiti* DNA load (as determined by lower real-time PCR thresholds) in skin and vaginal scrapings, illustrating the association between parasitic load and antibody response (Schares, Maksimov, et al., 2011).

The most probable reason for the loss of SC and VV cysts is their destruction by the immune system. As the host reacts to the cysts, they are circumferentially surrounded by several layers of eosinophilic and granulomatous inflammation to destroy them (Langenmayer, Gollnick, Majzoub-Altweck, et al., 2015). However, further studies are needed to investigate whether the immune response eventually leads to the effective destruction of every parasitic cyst and complete remission. As there are always cysts without surrounding inflammatory cells, there are indications for an effective immune evasion by *B. besnoiti*. Immune evasion is poorly studied, and immunosuppressive proteins and factors have yet to be determined. The thick secondary outer cyst wall could act as one of the means of immune evasion providing effective sequestration from activated effector cells. Further research regarding that topic is certainly needed.

In Herd-BbGer1, bulls were rotated between different subherds. The effect of this rotation on transmission dynamics between bulls and females is likely minor due to the initial high herd-seroprevalence. In every subherd, there were few negative and many positive animals, which could facilitate the transmission between females or females and bulls. Every breeding bull used in Herd-BbGer1 got infected and many of them shortly after introduction into the herd. During the summer period, six of nine bulls got infected. A recent study in an Italian herd also reported high incidences among bulls with twice as much seropositive animals within 1 year (Gazzonis et al., 2017). In contrast, in our study, serological incidence rates between bulls and females were nearly the same. The high disease prevalence in female cattle constituted a large reservoir for rapid infection via insect transmission and likely also favoured an infection via direct contact. Whether bulls are at an increased risk of getting bovine besnoitiosis is still not clear. In a cohabitation experiment, the bull remained negative despite frequent mating activity (Gollnick et al., 2015). Similar results were obtained in an Italian herd were mating with a seropositive cow did not enhance odds of infection in seronegative bulls (Gazzonis et al., 2017). Nevertheless, mating of seropositive with seronegative animals has to be regarded as a possible route of *B. besnoiti* transmission.

In conclusion, the endemic Herd-BbGer1 was characterized by a very high seroprevalence and significantly lower clinical prevalence. The clinical and serological incidence rates were high. The ongoing herd health scheme, in which females with severe clinical signs and bulls with clinical disease were rapidly removed from Herd-BbGer1, was not sufficient. To control the disease, an integral control programme could be a feasible option. Obviously, the overwhelming presence of subclinically, mildly and moderately infected cattle was enough to cause these high incidence rates. On the opposite, the clinical remission rate was higher than the clinical incidence rate leading to a reduction in clinical prevalence in the end. In Herd-BbGer1, there was a significant reduction in total cyst numbers and IFAT titres over the 4 years leading to clinical and serological remission in eight animals. Low cyst numbers and low IFAT titres were significantly associated with negative clinical outcome. Thus, in this situation in an endemic herd, estimating individual animal cyst numbers can aid to assess degree of infection and provide a prognosis regarding the possible fate of the respective animal.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

[Correction added after initial online publication on 18 January 2018: Legend for Supplemental Figure S2 was included.]

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