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Bovine paratuberculosis: results of a control plan in 64 dairy farms in a 4-year period

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ABSTRACT

Paratuberculosis is considered one of the most economically devastating infectious diseases of domestic livestock, and the most effective control strategy is a combination of 'test-and-cull' and on-farm biosecurity measures. In Italy, a Voluntary National Control Plan (VNCP) and guidelines have been introduced to reduce the impact of the disease, and farmers can voluntarily enroll in the control plan. The main aims of this study were: i) the description of the trend over a 4-year period on total, within-herd (WH) and between herd (BH) apparent seroprevalences observed in 64 dairy herds members of a mutual company located in Italy after the introduction of a proposed "Customized Control Plan" (CCP); ii) the evaluation of its effectiveness in terms of percentage of participating farms that decided to join the VNCP. Analyses on serum samples were performed with Enzyme-Linked Immuno Sorbent Assay (ELISA) method and revealed a general decrease in both total, WH and BH apparent seroprevalence. Total average apparent seroprevalence decreased from 2.39% in 2017 to 1% in 2020. Negative herds raised from 51.9% in 2017 to 71.1% in 2020, while farms with WH apparent seroprevalence > 5% decreased from 17.3% in 2017 to 4.4% in 2020. BH apparent seroprevalence decreased from 51.2% in 2017 to 29.2% in 2020. Among the 52 out of 64 herds that accepted to continue the proposed CCP after the first year, 41 (78.8%) joined in 2020 the VNCP, that assessed the health ranking of the herds. The results provide evidence that a control plan based on a farm-specific strategy and a subsidized testing process can effectively reduce the impact of paratuberculosis in dairy herds, especially in convincing farmers to continue in paratuberculosis control by joining the VNCP, including them in a National context and increasing their awareness of the disease.

1. Introduction

Paratuberculosis, also named Johne's disease, is an infectious disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), firstly diagnosed in 1895 in Germany (Johne and Frothingham, 1895). It is defined as a chronic and progressive granulomatous enteritis that affects mainly wild and domestic ruminants, but it can also infect horses, camelids, pigs, rabbits, badgers, stoats, foxes, weasels (Greig et al., 1997; Beard et al., 2001; Hutchings et al., 2010). It is spread worldwide with high prevalence: in a recent survey involving 48 countries, in about half of them more than 20% of herds and flocks were infected with MAP, with tendency to under-estimation (Whittington et al., 2019).

Because of MAP pathogenetic characteristics, such as orofecal transmission, high resistance in external environment, incubation period of 2–10 years (Whitlock and Buergelt, 1996) and long subclinical phase without signs of infection, paratuberculosis is considered one of the most economically devastating infectious diseases of domestic livestock. Economic losses are associated with reduced milk production (Beaudeau et al., 2007; Raizman et al., 2007a), low reproduction efficiency (Johnson-Ifearulundu et al., 2000; Raizman et al., 2007a;

Abbreviations: VNCP, Voluntary National Control Plan; WH, within-herd; BH, between-herd; CCP, Customized Control Plan; MAP, *Mycobacterium avium* subs. *Paratuberculosis*; EFSA, European Food Safety Authority; ELISA, Enzyme-Linked ImmunoSorbent Assay; PCR, Polymerase Chain Reaction; rtPCR, real-time Polymerase Chain Reaction, qPCR, quantitative Polymerase Chain Reaction.

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Garcia-Ispierto and Lopez-Gatius, 2016), increased incidence of mastitis (Pritchard et al., 2017; Rossi et al., 2017) and other diseases (Villarino and Jordan, 2005; Raizman et al., 2017b), premature culling, increased costs of testing and therapy (Benedictus et al., 1987) and decreased culled cow values in affected herds (Good et al., 2009). The economic impact of the disease is approximated to cause a total loss of 200 million ± 160 million $ annually in the United States (Losinger, 2005), and the European Food Safety Authority (EFSA) estimated that the total costs related with paratuberculosis in the European Union can reach 500 million €/per year (More et al., 2017). Furthermore, indirect losses related with paratuberculosis infection can come from trade restrictions (Kennedy et al., 2017). In addition, there is still uncertainty about the implication of MAP in human disease (More et al., 2017; Garvey, 2018;), for example the potential link with Crohn's disease (Chiodini et al., 2012), with more economic consequences related with consumers' perception of risk (Groenendaal, Zagmutt, 2008) or restriction to international trade of milk and milk products.

A MAP infection control program can require several years to determine a decrease of both within-herd (WH) and between-herd (BH) seroprevalence (Collins et al., 2010): the "test and cull" method is an effective strategy to decrease MAP prevalence (Nielsen and Toft, 2011; Kirkeby et al., 2016; Konboon et al., 2018) although, if applied alone, this will not eradicate paratuberculosis even after many years (McKenna et al., 2006; Dorshorst et al., 2006). Enhancing on-farm biosecurity, particularly for young animals, is another effective tool to reduce MAP prevalence. The combination of a 'test-and-cull' scheme and on-farm biosecurity measures (Dorshorst et al., 2006; Kudahl et al., 2011; Smith et al., 2017) is the most effective control strategy. Biosecurity measures should be considered also to control the transmission between herds and to protect MAP-negative ones, as the purchase of sub-clinically infected animals is considered the main factor of between-herd transmission (Rangel et al., 2015). In Italy, studies on prevalence in northern and southern regions showed that the disease is widespread (Pozzato et al., 2011; Marchetti et al., 2013). This has to be considered especially in trades involving non-EU countries like China and India that request importation of MAP-free products.

For these reasons, Italian Ministry of Health has developed a Voluntary National Control Plan (VNCP) and Guidelines (Italian Ministry of Health, 2013) aimed at controlling MAP and assigning health ranking to the herds. Farmers, advised by their veterinary practitioners, can voluntarily enroll in a control program that aims at gradually reducing the prevalence by adopting biosecurity measures coupled with an appropriate testing scheme, based on serological and fecal assays with Polymerase Chain Reaction (PCR) or culture. The VNCP will allow farmers to obtain a health certification with a ranking system based on apparent WH seroprevalence, in order to divide farms into different risk-based groups: farms with clinical cases; farms with no clinical cases and WH seroprevalence > 5%; farms with no clinical cases and WH seroprevalence < 5%; farms with no clinical cases and WH seroprevalence 0%. The present study had two main aims: i) to describe the trend on total average, WH and BH apparent seroprevalences observed in dairy herds members of a mutual company located in Italy, after the introduction of a MAP "Customized Control Plan "(CCP); ii) to evaluate the effectiveness of this CCP in terms of % of involved farms that joined the VNCP among the 4-year period.

2. Materials and methods

2.1. CCP design and sample collection

The study was conducted from January 2017 to December 2020, with a starting population of 64 dairy herds members of an Italian mutual company located in the Southern part of the country. The farmers were asked to voluntarily participate to the study, so they were not randomly selected. The herds were categorized in relation to their size, following sampling protocols of National Guidelines, considering cut-offs of 40, 50, 60, 100, 300 and 500 animals. The CCP was organized as follows: the 1st year (2017) was aimed at obtaining a complete overview about total average MAP apparent seroprevalence in all the 64 farms and at performing a risk assessment for each farm; 2nd, 3nd and 4th year (2018–2019–2020) aimed at evaluating the total average, WH and BH apparent seroprevalence in the farms that accepted to continue the CCP and to evaluate their trends along the 4-year period. Data obtained from dairy farms management evaluation and from serological tests were used in order to develop a farm-specific strategy to control MAP infection. Serum samplings of all the animals older than 24 months were performed by an accredited veterinarian at least once a year, twice if requested by the farmer. In order to give to the farmers the chance to prioritize the culling process, they could request a real time-Polymerase Chain Reaction (q-PCR) analysis of seropositive individuals suspected to be active shedder on feces.

2.2. ELISA testing

Serum samples were analyzed using a commercially available ELISA kit (IDEXX Screening Ab, USA) at the National Reference Center for Paratuberculosis of Piacenza. Positive and inconclusive results were subsequently submitted to confirmation by testing with another commercially available ELISA kit (IDEXX Confirmation Ab, USA), with an outcome expressed as positive or negative results. Both tests' procedures and interpretation of the results were performed in accordance with the instructions provided by the manufacturer.

2.3. DNA extraction and q-PCR

Q-PCR on feces was performed under specific request of the farmers, on seropositive individuals that were suspected to be active shedders with the aim to prioritize the culling. DNA extraction from fecal samples was obtained by suspending 1 ± 0,2 g of feces in 20 mL of sterile distilled water, vortexed, and allowed to settle for 5–20 min at room temperature. Then, 300 μL of the suspension were collected into tubes containing 200 mg of acid-washed glass beads (Sigma Aldrich, Milan, Italy) and 200 μL of sterile water for the bead-beating step in Tissue Lyser II (Qiagen, Milan, Italy) for 10 min at 30 Hz. 180 μL of Buffer AL and 20 μL of Proteinase K were added to sterile tubes containing 200 μL of samples. After 10 min of incubation at 70 °C, 210 μL of ethanol were added, and all the lysate obtained was loaded into a QIAamp Column placed in a collected tube. The columns were washed with 500 μL of Buffer AW1 and AW2, centrifuging the tubes at 16,000xg for one minute after the Buffer AW1 wash and at 16,000 x g for three minutes after the Buffer AW2 wash. Subsequently, the DNA was eluted in 200 μL of elution buffer and after 5 min centrifuged at 16,000xg for one minute. 200 μL of eluted DNA were then used for the PCR analysis. For the direct detection of MAP in fecal samples, IS900-quantitative PCR (qPCR) was performed. qPCR, primers, probes, and other PCR conditions were applied as previously described (Donaghy et al., 2011). Positive samples were classified as strongly positive (less than 28 Cq) or weakly positive (between 28 and 36 Cq) based on the Cq values.

2.4. Farm-specific strategy

In 2017, an initial risk assessment was performed in every involved farm through the application of the biosecurity checklist redacted by the National Reference Center for Paratuberculosis (Italian Ministry of Health, 2013). It allowed to highlight and correct the main management issues (including procedures and behaviors) that could represent a risk for MAP introduction and transmission. Considering seroprevalence, analysis of the biosecurity checklist results as well as PCR results, when performed, a farm-specific strategy was developed focused on culling of positive-tested animals and suggesting biosecurity measures that farmers could follow. Communication to each farmer was accomplished through videocalls in 2018. Every farmer was asked to choose between three options: i) to follow the proposed CCP and to join the VNCP starting from 2019 (in that case, serum samplings were then performed by veterinarians from the Local Health Unit, following the sampling scheme provided by National Guidelines); ii) to follow only the proposed CCP; iii) to not continue with the CCP (in that case, they were not considered part of the project anymore).

2.5. Statistical analysis

Descriptive statistical analysis was conducted considering apparent seroprevalences. Total average seroprevalence was calculated as the number of positive samples out of total serums tested; WH and BH apparent seroprevalence were calculated, respectively, as the number of serologically positive cows among the total number of cows tested, within one herd, and the number of herds with at least 1 serologically positive cow among the total number of herds tested for every year. Herds were classified as negative herds (0PH), when apparent WH seroprevalence was 0%; low prevalence herds when it was < 5% (LPH), and high-prevalence herds when it was higher than 5% (HPH), according to National Guidelines cut-offs (Italian Ministry of Health, 2013). A management issue analysis was also performed, with an initials identification and classification as follows: lack of the maternity pen; incorrect calving barn management; overcrowding; lack of the milking parlor; poor water management; inadequate hygiene of heifers/adults environment, purchase of replacements from herds without sanitary warranties and others. Suggestions were monitored on farms with at least one management issue. The prevalence of management issues was calculated in relation to herd size (farms with less than 40 cows, farms with 41–100 cows, farms with more than 100 cows). Fisher's exact test with Yates correction was used to detect the association between herd size and the presence of management issues, considering significant results at a p-value < 0.05.

3. Results

In the 4-year period, a total of 21,024 serum samples were analyzed, and 418 (1.99%) were classified as MAP positive. In 2017, 139 samples out of 5806, collected in all the 64 farms, were positive for MAP with a seroprevalence of 2.39%.

From the starting number of 64 dairy herds, 52 accepted to continue after the first year, 3 decided not to continue while 8 were excluded because they were forced to close. One farm accepted to be sampled every year but did not follow the proposed scheme, so it was not included in the study, however its apparent WH seroprevalence was 5.2% in 2017, 3.8% in 2018, 5.8% in 2019 and 7.6% in 2020. Out of the 52 herds that accepted to continue the CCP, 51 were sampled in 2018, 48 in 2019, 45 in 2020. This was due to other sanitary problems and/or COVID-19 restrictions. Considering the 4-year period, at least one sampling per year was performed in 41/52 (79%) farms.

Size distribution of the 52 considered herds are reported in Fig. 1 where it is noticeable that the majority of the herds (33/52, 63%) had less than 40 animals. Regarding the trend of total average seroprevalence, the percentage of positive samples progressively decreased from 2.39% in 2017 to 2.21% in 2018 (121/5482), 1.5% in 2019 (98/6366) and 1.0% in 2020 (32/3370). As regards the WH apparent seroprevalence, median apparent WH seroprevalence trends in relation with herd size are shown in Fig. 2, where is evident a general decrease trend regardless of herd size. Farms classified as 0PH (apparent WH seroprevalence 0%) were 51.9% of total herds (27/52) in 2017, then raised to 54.9% in 2018 (28/51), 56.2% in 2019 (27/48) and 71.1% in 2020 (32/45). When considering only farms with at least one sampling per year, % of 0PH increased from 48.8% (20/41) in 2017–70.7% (29/41) in 2020. Farms classified as LPH (seroprevalence <5%) were 30.8% in 2017 (16/52), 31.4% in 2018 (16/51), 29.2% in 2019 (14/48) and 24.4% in 2020 (11/45). HPH (farms with seroprevalence >5%) were 17.3% in 2017 (9/52), 13.7% and 2018 (7/51), then 14.6% in 2019 (7/48) and 4.4% (2/45) in 2020 (see Fig. 3). Considering farms with at least 3 samplings performed during the 4-year period (51/52), 23/51 farms (45.1%) maintained WH apparent seroprevalence to 0%; 12/51 (23.5%) were able to set WH apparent seroprevalence to 0%; 9/51 farms (17.6%) maintained apparent WH seroprevalence below 5%; 4/51 farms (7.8%) decreased apparent WH seroprevalence to < 5%. In 3/51 farms (5.9%) an increase of apparent WH seroprevalence was recorded. BH apparent seroprevalence was calculated in farms with at least 1 sampling per year (41/52) and it decreased from a starting 51.2% (20/41) in 2017–29.2% (12/41) in 2020. During the 4-year period, q-PCR was performed from 68 fecal samples of seropositive animals and 9 resulted positive (13.2%). Out of the 52 herds that accepted to continue the CCP in 2019, 31/52 farms (59.6%) decided to join the VNCP and in 2020 the number increased to 41/52 (78.8%).

The application of the "test and cull" scheme was accomplished in all participating farms, while for what concerns the management issues analysis, at least one issue was found in 26 out of 52 (50,00%) of the herds, of which fifteen (57,69%) were able to apply the suggested changes. The most common followed suggestion was in relation with inadequate hygiene of the heifer/adult environment (n = 8), followed by corrections of the calving management (n = 4). On the other hand, eleven farms (42,31%) were unable to follow the suggestions, and decided to follow only the "test and cull" scheme. In both cases, apparent WH seroprevalence was maintained < 5% or zeroed, except for

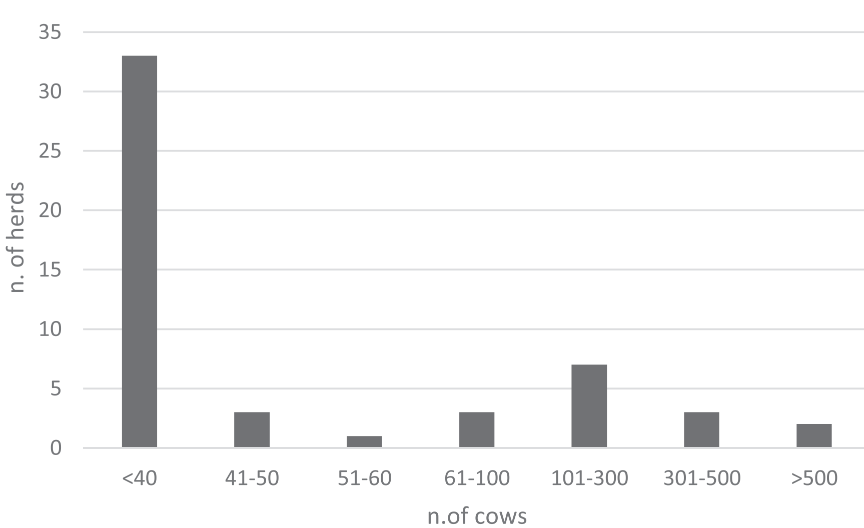

Fig. 1. Herd size distribution of participating herds. The number of cows for each herd is shown in the x-axis, while the number of herds for each range of cows is in the y-axis. 33 herds (63,5%) were composed by 40 or less cows; 3 herds (5,8%) were composed by 41–50 cows; 1 herd (1,9%) was composed by 51–60 cows, 3 herds (5,8%) were composed by 61–100 cows; 7 herds (13,5%) had between 101 and 300 cows; 3 herds (5,8%) had between 301 and 500 cows; 2 herds (3,8%) had more than 500 cows.

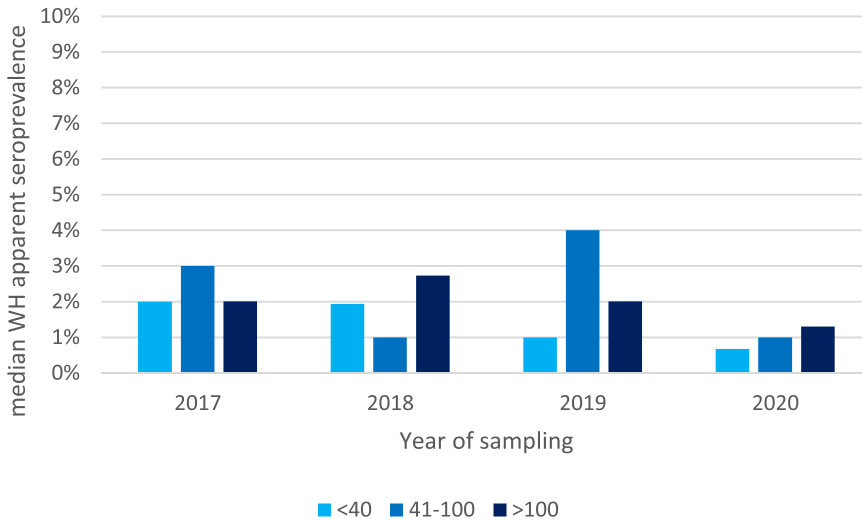

Fig. 2. Distribution of median WH apparent seroprevalence considering herd size during the 4-years period. Median WH apparent seroprevalence decreased in every group. In farms with < 40 cows it was 2% in 2017, 1,9% in 2018, 1% in 2019 and 0,7% in 2020. In farms with 41–100 cows it was 3% in 2017, 1% in 2018, then it increased to 4% in 2019 and decreased again to 1% in 2020. In farms with more than 100 cows it was 2% in 2017, 2,7% in 2018, 2% in 2019 and 1,3% in 2020.

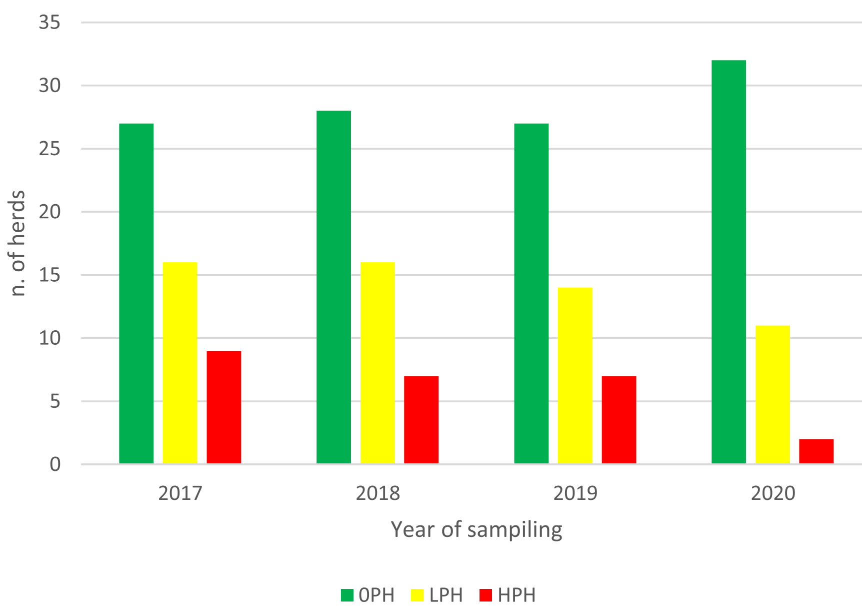

Fig. 3. Distribution of farms considering apparent WH seroprevalence cut-offs. Farms classified as 0PH (with apparent WH seroprevalence set at 0%) were 27 in 2017, 28 in 2018, 27 in 2019 and 32 in 2020, respectively. Farms classified as LPH (with apparent WH seroprevalence <5%) were 16 in 2017 and 2018, 14 in 2019 and 11 in 2020. Farms classified as HPH (with apparent WH seroprevalence >5%) were 9 in 2017, 7 in 2018 and 2019, 2 in 2020.

one herd in which an increase was registered in 2020. In that case, the farmer applied the suggested changes but also purchased animals from herds without a sanitary warranty in 2019.

Table 1
Distribution of the management issues analysis. No statistically relevant association with the herd size was detected.

Management issue	n (%)
lack of the maternity pen	6 (11,54%)
incorrect calving barn management	5 (9,61%)
overcrowding	4 (7,69%)
lack/problems of the milking parlor	1 (1,92%)
poor water management	3 (5,77%)
inadequate hygiene of the heifer/adult environment	10 (19,23%)
purchase of replacements from herds without a sanitary warranty	5 (9,61%)
others	1 (15,38%)

The distribution of management issues analysis is shown in Table 1. The most common issues were inadequate hygiene of the heifer/adult environment (n = 10) and the lack of the maternity pen (n = 6). No statistically significant association was found between herd size and the presence of management issues.

4. Discussion

The present work is the first in Italy designed to follow and to evaluate MAP apparent seroprevalence trends in the same herds during a temporal window, through the application of a CCP that aimed at involving farmers into the VNCP. Although data collection was troubled by other sanitary problems that occurred into some farms and COVID-19 sanitary restrictions, this study describes a general decrease in apparent seroprevalence indicators after the introduction of the CCP and demonstrates the effectiveness of this customized approach in terms of convincing farmers to enroll in the VNCP. Indeed, a CCP such as the one described in this study can be an effective tool to put in contact farmers and national institutions, helping both of them. On one hand, national institutions can add data to the national database, improving the general overview of the disease. Indeed, obtaining epidemiological data on MAP prevalence in different regions is crucial for the development of effective control programs. On the other hand, by joining the VNCP, farmers are assisted in the reduction of JD impact through annual checks, and also they can increase their awareness about the disease and be more collaborative. Furthermore, they have had the chance to obtain a health ranking based on WH apparent seroprevalence and consequently promote the conscious trade of animals and their products. The certification of the herd status by Veterinary Services is very important, allowing a transparent and safe procedure for between-farms trade of replacement animals, and for trade of dairy products. At the end of the study, 41/52 farms (78.8%) had joined the VNCP and obtained a health ranking.

The farm-specific approach implicates that farmers were constantly informed about the results and, without being forced, decided to collaborate since the surveillance plan created a feedback-based mechanism in which they could take decisions with a more complete overview of the epidemiological situation of the farms. In addition, it allowed to establish a strict communication flow between veterinarians and farmers, without any obligation that helped to achieve an effective and positive collaboration, even from an economic point of view. Indeed, although the test costs related with the diagnostic tests were completely covered by the mutual company of which the farms were members, the farmers covered the costs related with the culling process and the application of biosecurity measures, when they decided to follow the suggestions. This highlights how once the evidence of the economic and health benefits is shown to them, most of the farmers showed collaboration and decided to cover, even just partially, the costs of such plan. Nevertheless, the results from this study should be taken with caution and not applied *in toto* to other situations in which farmers are asked to cover all the costs related with the control plan. Indeed, the participation rate and the general results could be very different in contexts where farmers do not receive any kind of subsidization. Notably, this could be related with the fact that the application of the suggested biosecurity measures, not subsidized by the company, seemed a harder strategy to follow compared with the "test and cull" policy alone, for which farmers received economic help. This finding is in accordance with a Swiss study by Klopfstein et al. (2021), in which culling tested-positive animals was found to be the most followed proposed measure among 17 Swiss cattle herds, while other recommendations were rarely realized (38%). In our case, after the assessment of the main biosecurity issues, eleven farmers did not follow the suggestions but continued to cull positive individuals, with similar results to those who decided to apply the suggestions. Nevertheless, this could be due to the low number of included farms, and the application of just a "test and cull" scheme alone, even if aggressive, is not recommended (Crociati et al., 2022). Indeed, a good control plan should not only include the removal of already-infected individuals, but also the prevention of new infections (eg. from purchased animals), particularly in young individuals.

Further research should take this *modus operandi* as an example to convince farmers to join national control plans, possibly during a period longer than 4 years. Next studies should look at farms individually and with more detail, particularly in the evaluation of which farm-specific measures are more effective to be followed by farmers to achieve their compliance, especially from the perspective of convincing them to be enrolled into national control plans.

In other studies, between-herd and within-herd true prevalence estimates vary widely, although, the direct comparison is difficult due to the differences in the applied diagnostic tests, testing strategies, and sampling design (Garcia and Shalloo, 2015). Generally speaking, the majority of previous studies in Europe reported apparent or true MAP prevalence in different countries or regions in order to have an overview about the epidemiologic situation. On the other hand, this study wanted to follow the participating farms on a temporal frame to evaluate the effect of a "Customized Control Plan", not only in its ability to reduce MAP seroprevalence, but also in convincing farmers to enroll into the VNCP. A similar Danish study (Verdugo et al., 2015), conducted from 2011 to 2013 on dairy cattle farms participating at the Danish National Control Plan had similar results in terms of decreasing trend of MAP true prevalence at both herd and animal level, although the study was conducted on individual milk.

Dairy herds' owners participated in a voluntary MAP testing scheme, therefore our study may be subject to selection bias, because herds were not randomly selected. Despite this, Ritter et al. (2016) demonstrated that participation in a voluntary paratuberculosis control program was not associated with herd-level MAP prevalence. Data on apparent MAP seroprevalence in dairy cattle herds from Southern Italy not participating in the study remains unknown and should be implemented in future research. The one farm that decided not to follow the proposed CCP, but accepted to be sampled every year, showed an increasing apparent seroprevalence trend (from 5.2% in 2017 to 7.6% in 2020).

5. Conclusions

The results of this study confirm that paratuberculosis is a widely spread disease that affects a large proportion of dairy cattle herds. The application of a tailored, "customized" Control Plan based on biosecurity measures coupled with identification and removal of positive-tested individuals, can be able in the long period to reduce the impact of paratuberculosis, when subsidized testing is available, in terms of seroprevalence decrease (BH and WH), but especially by convincing farmers to enroll in the VNCP, increasing the awareness about MAP and allowing a better national overview of the disease.

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Declaration of Competing Interest

All the authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) the work. They all declare that there's no financial/personal interest or belief that could affect their objectivity.

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