CONTROL OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS INFECTION IN A DAIRY FARM IN ALBANIA

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ABSTRACT

Paratuberculosis is a chronic and contagious granulomatous enteritis. In cattle it is characterized by persistent diarrhea, and progressive weight loss (Whittington and Sergeant 2001). The *Mycobacterium avium subsp. paratuberculosis* (MAP) is the etiologic agent and, is excreted in large numbers in feces of infected animals and less in colostrum and milk. Fecal shedding begins before the appearance of clinical signs, and subclinical animals are important sources of transmission. Infected animals can appear healthy for months to years. The present paper reports the successful decrease from 2018-2021 of the seroprevalence and infection rates in a herd with MAP infection. A total of 1613 sera and 47 stool samples underwent RT PCR and microscopic investigation. Stool and blood samples were collected from animals of different age groups. The Real Time PCR employing TaqMan® MAP Reagents & Xeno™ DNA Controls and DNA amplification involving Applied Biosystems 7500 Real-Time PCR System was used to test the bacterial shedding of stool samples. The Ziehl-Neelsen method was applied for the direct detection of MAP in stained faecal smears. Individual serum samples tested via cELISA were used to detect antibodies against MAP (https://www.idexx.co.uk/en-gb/livestock/livestock-tests/ruminant-tests/idexx-paratuberculosis-verification-ab-test/). High percentage in positive results (6.8% seroprevalence, and 77% - 88% agent detection) show the possibility of a long-term infection persistence in this heard. Detailed analysis was necessary for the evaluation of positivity. In addition, management measures for the disease control were considered. Applying different laboratory methods is an added value to early detection of infected animals (Pinedo *et al*. 2008). Testing strategy accompanied by appropriate management measures in the herd show impressive decrease in the prevalence of infection.
Keywords: MAP, seroprevalence, Real Time PCR, cELISA, microscopy

1. INTRODUCTION

Animal production in Albania is considered one of the most important branches of the economy, because it's the main source of income for the rural population. Cattle are raised almost everywhere, even in remote mountainous areas. The official data of 2020 report that the population of cattle and calves is approximately 370 thousand, 300 thousand of which are dairy cows. In total, there are 122 thousand cow–calf herds which makes up almost 1/3 of herds in the country. Herds with over 100 cows are few, just several dozen. There are many and complex reasons behind the challenges the cattle breeding sector is facing. One of the most sensitive aspects in cattle breeding is the control and monitoring of diseases, especially those that cause economic damage to the herd, paratuberculosis or Johne's Disease is among of them.

Paratuberculosis is a chronic, and contagious granulomatous enteritis. In cattle it is characterized by persistent diarrhea, and progressive weight loss (Whittington and Sergeant 2001). Mycobacterium avium subsp. paratuberculosis (MAP) is the agent of John's disease (JD) in cattle. M. Avium subsp. paratuberculosis belongs to the species M. avium, subspecies: M. Avium subsp. Avium (synonym, M. avium, M. Avium subsp. Paratuberculosis (synonym, M. paratuberculosis) (Imirzalioglu *et al*, 2011). MAP lives in intestinal cells and lymph nodes, and it's excreted in large numbers in feces of infected animals and less in colostrum and milk (Collins 2021). Calves become infected usually within first month of age, but also during fetal life. Fecal shedding begins months even years before the appearance of clinical signs. The main source of transmission is subclinical animals that can appear healthy for months to years.

The present investigation aims to serologically, microscopically, and molecularly diagnose the MAP in dairy cows. The diagnosis tests were chosen based on the costs and number of samples. Direct diagnosis is based on the detection of MAP in feces using Real Time PCR (Stevenson 2010), and microscopic examination via Ziehl - Neelsen (ZN) stain method. For the detection of antibodies against MAP in individual serum, ELISA test is generally used. It is best used to determine the infection prevalence in a herd, and to detect infection in the later stages of infection, but also it's a rapid and low-cost method (Collins *et al*., 2006).

2. MATERIALS AND METHODS

2.1 Sample selection

Fecal and blood samples were collected from animals of different age groups of one of the biggest herds in Albania. These samples are used in regular basis to detect *Mycobacterium avium subsp paratuberculosis*. Fecal culture is considered more sensitive and specific, and pooling of fecal samples of individual animals may reduce the high costs of fecal culture (Van Schaik 2007).

Initially, the criteria used to determine the heard health status, was testing all animal. Intending strategy was to remove infected cows along with limited measures to protect vulnerable calves. In the first year blood samples were taken from all adult dairy cows and heifers. In third were included in sampling calves less than 6 months off age.

2.2 Samples preparation

Blood samples were collected in plastic tubes, without anticoagulants. The serum was separated in the laboratory and stored in congealment till testing time. From suspected cows or those with clinical signs of disease, fecal samples were collected. Fecal samples were collected in 200 ml plastic cups and transported to the laboratory under cooling condition. The fresh stool sample is stored for at least 1-2 hours at room temperature. Once brought to laboratory, the samples underwent microscopic examination and stored at -18°C for further examination.

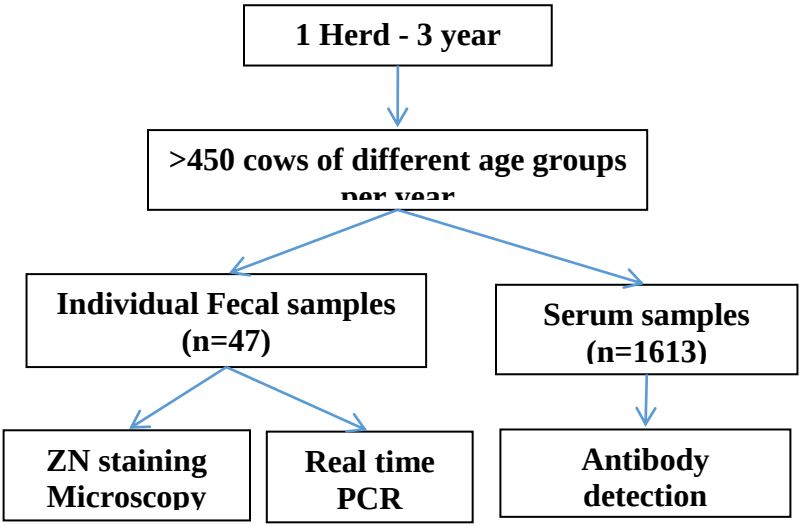

Fig. 1: Overview of study design.

2.3 METHODS

Microscopic investigation employing the Ziehl-Neelsen method determines the presence of mycobacteria directly in stained fecal smears. The method is based on phenol allowing fuchsine solution to penetrate inside bacterial cells. Once stained with fuchsine solution, some mycobacterium remained red even after the application of the acid-alcoholic solution, which means that this bacteria is acid-alcohol resistant. Other bacteria lose their red color and turn blue from the application of methylene blue solution.

Detection of MAP using RT PCR was carried out on individual fecal sample collected from suspected animals (Imirzalioglu *et al*., 2011). The total DNA is extracted using PSP Spin Stool DNA kit. Once extraction occurs, the master mix process involving the TaqMan® MAP Reagents & TaqMan® MAP and Xeno™ DNA Controls and DNA amplification using the Applied Biosystems 7500 Real-Time PCR System subsequently follows. The amount of specific target sequences present in a sample was calculated by measuring *ct* values and using standard curves generated with a series of known quantities of target sequences.

For the detection of antibodies directed against MAP in individual serum and plasma samples an ELISA test was used (Hirst *et al*., 2002). IDEXX paratuberculosis verification. microplates are coated in alternance with control antigen (-Ag) and with MAP antigen (+Ag). Once pre-incubated (30 – 120 min) with *M. phlei* extract to bind unspecific antibodies, the samples are transferred and incubated in the wells of coated microplates. Once the

unbound material is washed, the anti-ruminates antibody enzyme conjugate is added, which binds to any Ag-Ab immune-complex. The unbound conjugate is subsequently washed away and a substrate/chromogen solution is added. In the presence of enzyme, substrate reacts with the chromogen to generate a blue color. Upon addition of stop solution, a yellow color is generated. This color development is directly related to the amount of antibodies against MAP present in the test sample.

3. RESULTS

1613 cows blood samples were submitted to tests. 47 out of 1613 cows' blood samples underwent real time PCR and ZN staining for microscopy. ELISA test provided 6.8% positive results (31/450) in the first year. 7 out of 9 stool samples showed pigmentation via microscopic examination. All fecal samples regardless the microscopic data underwent real time PCR, and results showed 8 positive cases. In the second and third year number of positive animals decreased noticeably. The change in seroprevalence and direct detection of MAP is in the Table 1 reported.

Table 1. Positivity % of MAP in, using 3 different methods

	ELISA		MICROSCOPY		RT PCR	
Year	**Sample no./+**	**%Positive**	**Sample no.**	**Positive**	**Sample no.**	**Positive**
2018	450/31	6.8%	9/7	77.7%	9/8	88.8%
2019	468/29	6.1%	26/5	19.2%	26/7	26.9%
2020	695/7	1%	12/3	25%	12/4	33.3%

Regardless the method applied and the high number of animals of all age categories included, the total percentage of positivity is quite low in the third. Table 2 reports the positive relationship between agent and antibody detection**.**

Table 2. Comparing positivity % of MAP between methods

	ELISA		MICROSCOPY		RT PCR	
Year	**Sample no./+**	**%Positive**	**Positive**	**% in herd**	**Positive**	**% in herd**
2018	450/31	6.8%	7	1.5%	8	1.7%
2019	468/29	6.1%	5	1.0%	7	1.4%
2020	695/7	1%	3	0%	4	0.5%

4. DISCUSSION

MAP appears in a range of forms of disease with high prevalence. Detection of infected animals within a herd relies on use of different laboratory tests. A combination of laboratory tests to animals in different stages of the disease and would provide appropriate information about the prevalence of the disease in the herd. In addition, hygienic conditions and breeding in stables are necessary. The time factor and tests repetition are the greatest allies of MAP detection because of the disease progress and effectiveness of most of the tests in the later stages of the disease.

In the farm prevalence of infection decreased because the infectious pressure was reduced by culling of heavily shedding animals and the limited measures to prevent the calves from exposure to Mycobacterium avium subsp. paratuberculosis. Cows that resulted positive when tested with ELISA were separated from other animals and placed in a different compartment. All management and milking activities were separately carried out.

Other management measures for a higher protection of young animals through separation from infected cows and their colostrum would have reduced the risk of neonatal infection. Calves born from positive mothers were fed pasteurized colostrum and milk, or fed by other healthy cows in the herd. This was considered the most effective measure in reducing the risk of infection in newborn calves.

Shedding cows within a herd will be a source for the contamination of environment with MAP until they are removed from the herd, contributing to the presentation of new infections, if no control program is established. Quantitative use of ELISA to identify animals for selective culling or isolation in herd is a cost-effective strategy for disease control; lower ELISA values are associated with lower probabilities of infection and lower rates of fecal shedding.

Despite the importance of the cattle production, MAP in Albanian herds remains relatively unexamined, and very limited epidemiological information and data especially on molecular characterization of MAP are available. The present investigation is the first of its kind in Albania, and additional studies must further. Application of molecular methods allow for the identification of various MAP isolates present in our country, in addition to their genetic description and differentiation.

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