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Cattle Tuberculosis and Their Vectors: Tools for Early Detection of Biological Risks of the Disease[1]

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Abstract – Tuberculosis is a zoonotic disease affecting farm and wild animals as well as humans. Biological factors play an important role in the spread of this disease at the regional level. Control of occurrence and spread of tuberculosis at the territories requires the use of modern diagnostic technologies providing express identify the causative agent of tuberculosis followed by application of anti-epizootic measures to eradicate the disease. Our study aimed at searching the most highly effective tools for diagnosis of tuberculosis in different animal species. Different animal species produce antibodies to different mycobacterial antigens that do not allow creating a unique single antigen test system. In addition, at different stages of the disease animals react differently to the secreted and cell antigens of tuberculosis pathogen. It becomes apparent creation of multiplex test systems that allows analysing of biological fluids to multiple antigens simultaneously. We used the multiplex test system on cattle, goats, farm and wild deer, alpaca, badgers and other species. The multiplex serological immunoassay allows to explore of different animal species for tuberculosis and to detect the disease at different stages. The use of the multiplex test system will allow tuberculosis control at the regional level, with the possibility of exploring a variety of animals and promptly identify the pathogen reservoirs, potential risk factors of the disease.

Keywords – *tuberculosis; wildlife; multiplex immunoassay; mycobacterial antigens.*

1. Introduction

The biological risk factors associated with tuberculosis at the regional level includes not only mycobacterial biopathogens (*Mycobacterium tuberculosis, M.bovis, M.caprae*), but also involved in epidemiology of the disease host and vector animals. Farm animals and wildlife play an important role in epidemiology of the disease, especially when the biopathogen is *M.bovis*. Tuberculosis bioagents in wild/domestic animals can be transmitted to other wild animals, domestic animals, humans at the affected areas. Timeous detection of disease agents meets some challenges, such as missed or delayed disease outbreaks, capture and testing may injure or kill animals (wildlife). Most tests validated for domestic animals, but not for wild animals. However to reduce risks of tuberculosis to domestic animals, humans, and to conserve highly valued wildlife populations management of the disease in wildlife as well as in livestock is required.

Worldwide-used the single intradermal comparative tuberculin skin test remains high effective assay for detecting cattle infected with *M.bovis*; however it misses some animals with the disease (Whelan et al., 2011: 500). The control bovine tuberculosis is difficult when only the skin test is used. Another test for measuring cell-mediated immune response to *M.bovis* infection is IFN-ɣ assay which in conjunction with the skin test has improved diagnosis of tuberculosis but some infected ani-

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mals still go undetected. PCR methods can be used for indication of mycobacterial pathogens as post-mortem test (Khammadov et al., 2009: 34). To improve tuberculosis eradication programs in cattle farms other type of tests are needed, serological tests which can detect the specific antibodies to mycobacterial antigens can be very useful for that (Waters et al., 2011: 1887). Using the skin test in goats for the same purposes meets other problems, such as very thin skin in some breeds and in young animals. Improved blood (serum) tests are required to improve the control tuberculosis in goats (Shuralev et al., 2012: 296).

The spread of tuberculosis is strongly linked to specific wildlife reservoirs in many countries worldwide (Miller et al., 2013: 1362; Palmer et al., 2012: 12; Rodríguez-Prieto et al., 2012: 148). The implementation of monitoring programs using diagnostic tools with high sensitivity and specificity is the key to aiding eradication in wildlife reservoirs. Wild boar appears to be a wildlife reservoir of *M.bovis* and *M.caprae* infection in some European countries (Byrne et al., 2012: 1). Badgers have been identified as a significant source of *M.bovis* infection and have been implicated in the spread of the disease to cattle in UK, Ireland and other countries. Currently available diagnostic tests, such as the tuberculin skin test, are impractical for badgers because of the requirement for recapture. Developing effective diagnostic tests to aid the surveillance of the disease in the badger population is needed. Tuberculosis infection in South American Camelids like alpacas and llamas may not show overt clinical signs until the infection has reached advanced stages, and then can present with a sudden onset of symptoms and rapid mortality. There is a need to improve ante-mortem diagnosis in Camelids as the currently applied the single intradermal comparative tuberculin test can have low sensitivity with many animals escaping its detection (Lyashchenko et al., 2011: 2146, Rhodes et al., 2012: 1683). Other reservoirs for tuberculosis are wild and farm deer, which can re-introduce the disease to non-infected areas and other animals such as cattle as well as being affected by the disease themselves and other wild animals. Although the current standard tuberculosis diagnostic test for captive deer is the tuberculin skin test, the reported low specificity highlights the need for further development of better diagnostic tests (Queiros et al., 2012: 327). The problems associated with administration of the tuberculin injections due to the thin skin of some deer and false positive results as well as anergy observed in true positives adding to the difficultly in disease control are also occurred. Tuberculosis diagnostic tests are not available for many wild and some domestic animals. Dogs and cats usually are not tested for tuberculosis. However some recent reports indicate the possibility of these species to be infected with mycobacteria in affected urbanistic areas and as a result to be involved in epidemiology of tuberculosis. All potentially involved in the epidemiological process animal species can be biological risk factors of the disease. Prediction of biological risk factors for tuberculosis depends on the timely detection of the reservoirs in the wildlife and agricultural sector, what can be achieved by using appropriate diagnostic tools.

2. Methodology

Using a panel of specific *M.bovis* antigens as a tool for potential use in tuberculosis eradication a multiplex chemiluminescent assay was developed. Over 70 antigens (recombinant proteins and synthetic peptides) were investigated and extensive assay optimization has been done. The diagnostic tools for a range of species and tuberculosis vectors from families including Bovidae (cattle and goats), Cervidae, Suidae, Mustelidae, Camelidae, and other were developed and validated. The test system can be very useful in prediction of biological risk factors of the mycobacterial diseases.

2.1. The technology

The test system allows for different analytes multiple analyses to be obtained from a single serum sample within one microplate well. The platform is similar to a 96 well enzyme-linked immunosorbent assay (ELISA) format and offers high-throughput which makes it ease of use. Up to 25 separate antigen or antibody spots can be printed in each well. These spots are resolved and can be individually analysed. Therefore up to 2,400 individual data points can be returned from one standard ELISA plate. Parallel processing of biofluid samples using this technology is a huge leap forward for diagnostic laboratories. The assay system uses enhanced chemiluminescence technology to generate signals. Each spot has a range from 0 – 65,500 Relative Light Units and this dynamic range gives the potential to significantly improve the sensitivity of tuberculosis diagnostic serological tests.

2.2. Application

The application of the immunoassay is for the detection of *M.bovis* and other mycobacterial infection in primarily bovine samples but also the system is being optimized for other non-bovine species, such as wild boar, deer, goat, badger, etc. The incidence of bovine tuberculosis in countries around the world is of major concern in cattle as well as multiple wildlife species. There are a number of difficulties associated with the disease that have impacted the development of a suitable diagnostic tool. Multiple research reports have emphasised that the development of a diagnostic assay for tuberculosis will require the use of a multiplex system that can simultaneously detect and analyse antibody profiles to multiple antigens in a serum sample within a robust rapid system. One of the main difficulties in developing such a diagnostic tool was the identification of a suitable panel of *M.bovis* specific antigens that gave the sensitivity and specificity needed. The assay is rapid and data is analysed using a program that determines the result based on data from the panel of antigens. If this technology is implemented it could facilitate a reduction in bovine tuberculosis reactors, help eradicate the disease and provide a tool for the surveillance of *M.bovis* in various wildlife reservoirs.

2.3. *Mycobacterial antigens*

The antibody profiling to mycobacterial antigens in blood serum of tuberculosis affected animals depends on the stage of infection, animal species and pathological agent strains, individual immune system reactivity of macroorganism, and other things and conditions. Therefore several antigens should be used in a multiplex format considering mentioned conditions. Different antigens were investigated during this study, which are associated with M.bovis locus tags: Mb1598A, Mb1599, Mb1606c, Mb2057, Mb2659c, Mb2898, Mb2900, Mb3646c, Mb3902, Mb3903, Mb3904, Mb3905, Mb3909c, etc. Depending on animal species the serum samples are from, different combinations of the antigens are used for diagnosis of tuberculosis. For this purpose antigens were prepared as recombinant proteins, as synthetic peptides of immunogenic epitops of the antigens, or as protein fractions of supernatant or microbial cells revealed by electrophoretic fractionation in polyacrylamide gel.

3. Results and discussion

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3.1. *Domestic animals*

Cattle play important role in bovine tuberculosis infection agent of which is *M.bovis*. The tuberculin skin test identifies most infected animals, however some it misses some individuals. Serum samples from 60 cattle from tuberculosis affected herds were selected for the study. The animals were confirmed by histopathology/culture to be infected with *M.bovis* but results of standard skin test were negative (n=26) or difficult to evaluate (n=34, inconclusive). 92.3% and 85.3% of these animals respectively were identified in multiplex test-system as positive. The results indicate that the level of detection of *M.bovis* infected cattle can be greatly improved by the combined use of the skin test and the serological multiplex assay. Animals reacting negative in the standard tuberculin test represent potential biological risk factors of tuberculosis. The risk can be reduced using multiplex test-system in which these animals classify as positive by detecting specific antibodies to mycobacterial antigens. The need of new methodology for diagnostics of tuberculosis is one of the biggest issues in Russian Federation (Gulukin et al., 2012: 3) and the World.

According to the Veterinary Center of Russian Federation (Epizootic situation on severely hazardous animal diseases in the Russian Federation) for the period 2009-2012 tuberculosis in cattle was recorded in 7 federal districts of Russia: Central, Southern, North Caucasian, Volga, Ural, Siberian and the Far Eastern, including 9 federal subjects of the Central Federal District, where 2265 animals were infected (in Belgorod (855), Bryansk (144), Kursk (443), Lipetsk (35), Moscow (2), Orel (430), Ryazan (109), Tambov (76), Tula (171) oblasts); 4 federal subjects of the Southern Federal District, where 140 animals were infected (in Kalmykia (80), Krasnodar Krai (5), Volgograd (8) and Rostov (47) oblasts); 5 subjects of the North Caucasian Federal District, where tuberculosis was detected in 530 animals (in Kabardino-Balkaria (11), Ingushetia (12), North Ossetia-Alania (53), Chechnya (116), Stavropol Krai (338); 5 subjects of the Volga Federal District, where 4105 animals with tuberculosis were detected (Mordovia (1156), Tatarstan (2324), Saratov (211), Ulyanovsk (345), Orenburg (69) oblasts); 2 subjects of the Ural Federal District, where 177 animals were infected (Tyumen (2) and Chelyabinsk (175) oblasts); 5 subjects of the Siberian Federal District, where tuberculosis was detected in 860 animals (Altai Krai (146), Krasnoyarsk Krai (171), Irkutsk (127), Novosibirsk (400) and Omsk (16) oblasts); in the Far Eastern Federal District tuberculosis registered in Amur oblast among 93 animals. Thus, over the period 2009-2012 cattle tuberculosis registered in 31 subjects and 7 federal districts of Russian Federation in 8170 animals.

Goats constitute a biological risk factor associated with *M.bovis* and *M.caprae*. There is a need to implement a goat tuberculosis control program to ensure food safety. The multiplex chemiluminescent immunoassay system was optimized to enable the detection of an *M.bovis* infection in goats. A total of 495 goats were recruited into the study. Among the tuberculin skin test positive reactors the serological assay detected 177 of 180 animals. All goats from tuberculosis free area were negative in the multiplex immunoassay. The results suggest that a serum based assay for the detection of an M.bovis infection in goats is a feasible alternative to the intradermal tuberculin test which may cause quite painful reactions in tuberculosis infected goats and is not always easy to administrate with accuracy in younger goats with very thin skin.

Some other domestic animals such as sheep, pigs, dogs, cats do not play significant role but can be involved in epidemic process of tuberculosis and thereby may be in the line-up of biological risk factors. An optimization of the multiplex immunoassay to these vectors is under process.

3.2. *Wildlife*

Badgers act a wildlife maintenance reservoir for *M.bovis* and their close proximity to cattle facilitates transmission

of infection. Thus they increase the risk of tuberculosis in various species of animals in contaminated territories. The tuberculin skin test is impractical for badgers because of the requirement for recapture. Therefore, there is a need to develop effective diagnostic tests to aid the surveillance of the disease in the badger population. A total of 200 wild badgers were recruited from areas of high bovine tuberculosis prevalence. Post mortem and bacteriological examination of animals allowed dividing them into two groups: 67 infected badgers and the remaining 133 negative animals. The sensitivity of the multiplex assay was determined to be 56.7% using the culture results as gold standard. The apparent specificity was determined as being 97.0%. These results demonstrate that the multiplex serological assay is a valuable diagnostic test for tuberculosis in badgers and could help with the surveillance and control of disease.

Tuberculosis infected llamas may not show signs until the infection has reached advanced stages, and then can present with sudden symptoms that include coughing, respiratory problems, weight loss, etc. Therefore the need to diagnose infection in llamas at early stage of the disease is of importance. The use of a blood based test can add advantage of shorter turnaround times for results, giving potential to remove infected animals from herds faster to help reduce the risk of animal-to-animal transmission. 17 from 19 post-mortem confirmed tuberculosis positive animals had specific antibodies to investigated mycobacterial antigens, which were detected in the multiplex immunoassay. However, the specificity of the test still needs to be fully evaluated.

The population of wild boar appears to be a wildlife reservoir for tuberculosis caused by *M.bovis* and *M.caprae*. Preliminary results of the multiplex assay for a set of wild boar samples from Spain (102 culture confirmed negative, 44 *M.bovis* and 10 *M.caprae* positive animals) showed the sensitivity of 72.7% for *M.bovis*, and 60.0% for *M.caprae* infections and the specificity was 97.1%. Serum samples from 49 wild boars of Tatarstan region (Russia) were tested. None of them produce antibodies to mycobacterial antigens. That indicates that wild boars are not involved in epidemiological process in that region. However wild boar can be infected through transmission from cattle with tuberculosis positive status, and if it will be happen the spread of infection will be difficult to stop in wildlife of Tatarstan forest areas. Use of this serological test can greatly facilitate the monitoring of wild boar tuberculosis; thereby the biological risk factors can be controlled by avoiding the spread of this infection through trade and relocations. The performance of the test-system will be improved with further development and validation.

Currently for diagnosis of slowly progressive *M.bovis* infection affecting the Cervidae family tuberculin skin testing is used; however reports of test accuracy concerns highlight the need for alternative diagnostic tests for tuberculosis. Various Cervidae species and subspecies (total 779 samples) were investigated, including red deer, elk, white-tailed deer, reindeer, and fallow deer, which involved in epidemiology of disease as biological risk factors. The multiplex assay detected 86.8% in total of potential tuberculosis positive animals and 98.3% of deer with confirmed tuberculosis free status were negative, showing that this test is a reliable diagnostic tool for *M.bovis* in Cervidae. Potential to exploit this to increase sensitivity of diagnosis and use of combined cell-mediated immunity and serological based tests could lead to a highly effective approach for control and eradication of disease in deer.

Tuberculosis biological risk factors are associated with other wildlife species, such as Spanish ibex, mouflon, brushtail possum, fur seal, etc. Development of highly effective diagnostic assay is needed in multiplex format, which allow to test animals against several mycobacterial antigens and thus to control the disease spreading at the regional level. Our team continues its work on this field of research.

4. Conclusion

Prediction of tuberculosis biological risk factors will be successful if it is backed by appropriate diagnostic methods and techniques. The multiplex immunoassay is unique method for these purposes, it enables testing of blood serum samples against multiple mycobacterial antigens, it can be adapted not only to explore of various animal species but also human; analysis is performed easily and quickly

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