UNIVERSITY OF AGRONOMIC SCIENCE AND VETERINARY MEDICINE OF BUCHAREST

FACULTY OF VETERINARY MEDICINE

THE CONSEQUENCES OF INFECTION WITH *BRUCELLA MELITENSIS* AND CONJUNCTIVAL IMMUNIZATION BY REV1 STRAIN ON SAFETY OF SHEEP AND GOAT MILK

PhD Thesis advisor: Prof. univ. dr. DANEȘ DOINA

PhD Candidate: SHMEGLIA (AWWAD) ELENA

ABSTRACT

KEYWORDS:

Bacterioses, zoonoses, epidemiology, brucellosis, small ruminants, sheep, goats, laboratory diagnostics, molecular biology, phylogeny.

The PhD thesis "Veterinary Medicine" entitled "Consequences of *Brucella melitensis* infection and intraocular immunization with Rev1 strain on the safety of sheep and goat milk" was conducted at the University of Agricultural Sciences and Veterinary Medicine, Bucharest, at the Department of Veterinary Medicine Infectious Diseases and Preventive Medicine at the Faculty of Veterinary Medicine, Bucharest, under the guidance of Mrs. Prof. Univ. Dr. Doina DANEȘ.

The first objective of the study was to characterize the epidemiological situation and the risk of brucellosis in Palestine after a period of 15 years of implementation of the brucellosis control project using the vaccination of animals against this disease as the first tool. The second objective of this thesis was to characterize phenotypically and genotypically the wild strains of *Brucella melitensis* isolated in Palestine through classical and modern molecular biology techniques. The doctoral thesis is structured into two sections: the first section is devoted to the study of knowledge in the field, and the second is devoted to personal research. These sections are organized in three chapters, the first chapter presenting information from the literature on the subject of the thesis, which are used for the interpretation and comparison of the results of their own researches. Chapters II and III present the results of personal research.

CHAPTER I titled "BIBLIOGRAPHIC DOCUMENTING" summarizes the fundamental scientific information on the following aspects: general and morphological characteristics of *Brucella* genus, major antigenic determinants, pathogenicity, molecular determination *Brucella* and characterization of *Brucella melitensis* Rev 1. A subchapter is dedicated the nature of the brucellosis zoonosis, and this infection is described in animals and humans. Also, in this chapter are presented the bibliographic information about the laboratory diagnosis of brucellosis infections, from the collection of biological samples and to the current laboratory methods used.

CHAPTER II of the thesis presents the results obtained in the "Personal Research" on "RISK FACTORS ASSOCIATED WITH THE PREVALENCE OF MELITENSIS BRUSHES IN OVA AND BABY MILK AND ESTIMATION OF THE EPIDEMIOLOGICAL SITUATION OF BRUCELLOSIS IN CISIORDANIA PART OF PALESTINE", in two subchapters. The first subchapter "PRESENTATION OF PALESTINE AND THE MINISTRY OF AGRICULTURE" makes a short description of Palestine, Palestine, West Bank, Palestinian Government, Ministry of Agriculture - vision and challenges, interference with Israel in agriculture and the Palestine Animal husbandry, social characteristics in which Bedouins are an important element in livestock breeding, main sheep and goat breeds in Palestine, traditional dairy products in Palestine (fresh sheep and goat milk, jibnah baida, naṅngusia brænza), production of labna cheese (cheese-yoghurt), production of dried yoghurt (jameed, laban kisheq). At the end of this subchapter, Palestinian veterinary services are presented. The second subchapter, titled "BRUCELOSE IN PALESTINE AND RISK FACTORS", presents the studies conducted to investigate four major risk factors that are related to brucellosis in Palestine.

The first risk factor is investigated "ASSESSMENT AND REVIEW OF BRUCELLOSIS CONTROL: EPIDEMIOLOGICAL SITUATION BETWEEN 1974-2014 IN CISIODRANIA " were selected and consulted relevant official documents issued by the Ministry of Agriculture and submitted materials and methods that were used to control brucellosis in Palestine during 1974-1998 and following the implementation of Palestinian brucellosis control control brucellosis (PBCP) in 1998. The progress consecutive PBCP implementation was quantified by performance goals, such as: registration of farmers, animal vaccination, monitoring of vaccinated animals during the vaccination campaign, epidemiological-surveillance and monitoring of brucellosis control program, capacity building of veterinary services, capability development laboratory for brucellosis diagnosis, epidemiological-surveillance of *Brucella abortus* infection in dairy cows, awareness of target audiences and measure the impact of actions undertaken by KAP study. The investigations in this chapter assesses the impact PBCP the epidemiological situation of brucellosis after 15 years of project implementation, the prevalence of brucellosis in animals and humans, the impact PBCP other aspects of life and, finally, the critical issues that affect the result of the control program and conclusions.

The second risk factor investigated is to identify the frequency of *Brucella* infection by "PREVALENCE OF ANTI-*BRUCELLA* ANTIBODIES AMONG OTHER CASES ABORTIONS IN SMALL RUMINANTS IN CISIODRANIA - SEROLOGICAL INVESTIGATION" using materials and methods (sampling, the performance of the ELISA method, statistical analysis). *Brucella* abortion showed ranks third in the etiology of the abortion prevalent in small ruminants.

AN INVESTIGATION OF BRUCELLOSIS KNOWLEDGE, ATTITUDE AND PRACTICE AMONG LIVESTOCK OWNERS IN THE WEST BANK " is the third risk factor investigated using specific materials and methods (area of study, identification target population, ethnic considerations, questionnaire design, response processing and statistical analysis). The result of this study KAP - Knowledge, attitudes and practices, assessed by reference to data provided by the Palestinian Ministry of Health in 1999 and public awareness activities conducted by brucellosis control program 1999-2010.

The fourth risk factor was represented by the available diagnostic techniques and their performance, presented in the chapter "IMPROVING THE DIAGNOSTIC INSTRUMENTS USED IN THE CONTROL OF BRUCELLOSIS", a topic dealt with in two subchapters.

The first subchapter, " VALIDATION RT-qPCR TECHNIQUE FOR DETECTION OF *BRUCELLA* GENOME IN MILK SHEEP AND GOAT IN WEST BANK, PALESTINE " presents the RT-qPCR principle and the requirements to be met for validation of RT-qPCR techniques for sensitivity and specificity, limit of detection, linearity, y-intercept and coefficient of determination, coefficient of correlation, standard deviation and coefficient of variation, efficacy and repeatability, and calculus of the number of copies of the genome. This study also include samples processing (control strains, reference strain isolation, DNA extraction, *Brucella melitensis* vaccine Rev 1 DNA, RT-PCR amplification) and RT-qPCR validation by specificity and sensitivity, LOD and standard curve preparation, repeatability, reproducibility and test efficiency, robustness and gel electrophoresis. The conclusion of this study showed that RT-qPCR "in-house" is an inexpensive and accurate method for rapid detection of infectious agent and useful for rapid implementation of measures during the outbreak which help to prevent the spread of the disease and avoidance of human infections.

The second subchapter of investigation in the risk factor four is “DETECTION *BRUCELLA* GENOME BY RT-qPCR IN MILK SAMPLES OF SMALL RUMINANTS IN THE WEST BANK AS IMPORTANT TOOLS OF ADMINISTRATION THE OUTBREAK" showed how the protocol developed "in house" used during the outbreak of brucellosis and being presented, materials and methods, inclusively criteria the sampling of milk samples. They discuss the results and performance of the serological test Rose Bengal, the isolation of bacteria from milk samples, compared to the performance of RT-qPCR. The conclusion of this subchapter showed that’s the introduction of rapid, accurate and sensitive as the RT-qPCR technique is an extremely beneficial and provides a reliable tool for use by authorities in order to implement preventive measures to control outbreaks and to prevent the spread of disease among the human and animal populations.

In Chapter III is presented the "PREVALENCE OF INFECTION WITH *BRUCELLA MELITENSIS* AND IDENTIFICATION OF GENES WHICH CODIFY PATHOGENITY DETERMINATIONS ASSOCIATED WITH CELLULAR SURFACE IN FIELD STRAIN ISOLATED FROM CISIODRANIA, PALESTINA", the objective of which was answered by four studies.

The first study investigated the "MOLECULAR BASE OF VIRULENCE GENES OF

BRUCELLA MELITENSIS STRAINS ISOLATED IN CISIODRANIA, PALESTINA". This study is structured in materials and methods (harvesting and sample cultivation, PCR analysis, and identification of virulence genes through PCR), and presents results and conclusions that, despite the introduction of mass vaccination in Palestine in 1999, the presence of genetic determinants of pathogenicity associated with cells walls record an extremely high rate (95-100%) of strains isolated from the West Bank. The second study "GENETC POLYMORPHISM AND HOMOGENITY ANALYSIS OF *lps*B, *dac*E AND *ami*C CELL ENVELOP GENES AMONG WILD STRAINS OF *BRUCELLA MELITENSIS* ISOLATED IN CISIORDANIA, PALESTINA". Materials and methods are presented by sample harvesting and cultivation, primer design and PCR analysis, BLAST sequencing and BLAST analysis of *lps*B, *dac*E and *ami*C genes, homology and phylogenetic analysis, results (bacteriological analysis, gene sequencing and BLAST analysis) and conclusions. According to this study, the isolated strains of the West Bank were closely related *to Brucella melitensis* biovar 1, reconfirming the results of their identification by bacteriological methods based on phenotype, namely the morphological and biochemical properties.

In the third study is investigated the "EPIDEMIOLOGICAL APPROACH OF BRUCELOSIS THROUGH PHYLOGENETIC ANALYSIS PATTERN IN PALESTINE ". Materials and methods are presented (sample collection and bacterial isolation, primer design and PCR identification, primer and amplification protocol design, sequencing of amplification products, phylogenetic alignment and analysis), the result of alignment of sequences and the resulting phylogenetic tree, and finally the conclusions which shows that although the 18 isolates of *B. melitensis* have a common ancestor, but there has also variations between them. Sequences in the same district had a high degree of homology, but a higher degree of homology of genetic variation had evidence from the same villages, which led to localization on the same branches of the phylogenetic tree. This study showed that the close relationship between sequence variations depends on the distance between the sample of origin. One of the strains was identified as having a higher degree of homology with *the Brucella melitensis* Rev 1 vaccine strain, suggesting a possible infection by the vaccine strain. Worldwide phylogenetic mapping has revealed that the Palestinian strains are genetically related to those isolated from other Mediterranean basin countries such as Spain, Israel, Cyprus and Turkey, as well as those isolated in some Asian

countries such as India, Malaysia, and Indonesia. This finding indicates a common ancestral evolutionary origin of isolates.

In the 4th study, " THE PREVALENCE OF *BRUCELLA MELITENSIS* FIELD STRAIN ISOLATED FROM SHEEP AND GOAT AND MOLECULAR DISCRIMINATION OF *B. MELITENSIS* WILD VERSUS REV1 BY RESTRICTION ENZYME POLYMORPHISM (PCR-RELP) ANALYSIS OF *OMP*2 GENE " include materials and methods (isolation of the Brucella melitensis field and Rev 1 strains, PCR analysis, DNA digestion with the PstI enzyme and electrophoretic migration of digestion products). The results obtained showed that the DNA fragments obtained from the *B. melitensis* Rev 1 strain and two wild isolates from the flocks of the Hebron and Ramallah districts had a digestion profile similar to vaccine strain and resulted in three bands after digestion with PstI: 282 bp of the amplified *omp*2a gene lacking the PstI restriction site and two fragments smaller than 238 and 44 bp obtained from digestion of the amplified *omp*2b fragment. In contrast, *B. melitensis* wild isolates produced only two smaller fragments: a fragment of 238 bp and 44 bp.

Chapter IV of the thesis "NEW TRENDS IN THE LABORATORY DIAGNOSIS OF BRUCELLOSIS IN PALESTINE" brief to aggregate all the techniques of diagnostic laboratory were used for the research presented in the thesis and presents the overall design of diagnosis of brucellosis in Central Veterinary Laboratory of Palestine, the way of harvesting, collection and processing of the samples, the serological examination of serum samples, the assay of complement fixation (CFT), the indirect enzyme immunoassay (ELISA), the isolation and identification of *Brucella* sp, the molecular diagnosis of brucellosis - DNA extraction and identification by PCR PCR using one or more pairs of primers, the PCR real-time, the detection of genetic polymorphisms gene omp2 by PCR-RFLP, the sequencing of the amplified product, the alignment and the phylogenetic analysis, which have led to the conclusion that the authorities have provided the entire panel of diagnosis methods required for the brucellosis control.

The work ends with 19 general conclusions, 12 recommendations and with the bibliographic references.