SECRETARÍA NACIONAL DE EDUCACIÓN SUPERIOR, CIENCIA TECNOLOGÍA E INNOVACIÓN

PROYECTO PROMETEO

RESEARCH PROPOSAL FORM

Researcher's name	Said	Researcher's last name	Amer
Area of research	Molecular Epidemiology		
Title of PhD.	Humoral and cellular immune responses during *Trypanosoma evansi* infection in mice	University that awarded the Ph.D.	Tanta University, Egypt
Host institution (MAIN)	Technical University of Machala	Name of the counterpart in the host institution	Dr. Favián Maza
Higher Education Institution	NOT APPLIED	Name of the counterpart in the host institution	NOT APPLIED
Name of research with which the research contributes	Molecular Epidemiology of Bacterial Mastitis in Beef Cattle in El Oro Province, Ecuador: Economic Impact and Control Measures		
Objective of the research	The present study aims for accurate treatment and control of mastitis disease in beef cattle in El Oro Province, Ecuador via: (1) accurate identification of mastitis microbes through standard bacteriological procedures; (2) recommendation use of antimicrobial agents based on sensitivity/resistance to a wide panel of antimicrobial agents; (3) Determination of molecular diversity of mastitic bacteria and inferring their phylogenies; (4) statistical determination of factors interplayed on occurrence of mastitis; (5) elucidation of presence/absence of resistance genes in individual microbial strain/isolate; (6) determination the significance of recovered microbes as a public health concerns; (7) build up a risk factors analysis to help decision makers to forward strategic plans of veterinary and public health; (8) design a farm (herd) based management regimen to help control of the disease and increase farm sustainability and profitability		
Starting date of activities	15-Mayo-2014 27-Abr-2015	**End of activities**	12-Enero-2015 26-Agosto-2015
Total months of linkage	12 months		

Researcher Profile
The candidate is specialized in molecular epidemiology, with especial interest in zoonotic pathogens of which can transmit to human. Training in National Institute of Animal Health, Tokyo University of Agriculture and Technology and Tohoku University in Japan, as well as Chinese Academy of Science, China and CDC, Atlanta, USA provided opportunities to get acquainted with the state of the art in the field and learn from the first hand experts. Experiences and list of publication in the peer reviewed journals indicate that the candidate can work with any topic in the field of molecular epidemiology.

- In a concise way and no more than two thousand (2,000) words, please specify the following elements of your research proposal:

1.**Research Question and their delimiting spatial, temporal**

Intensive farming of livestock is associated with prevalence of several diseases which has veterinary and public health concerns, rather than significant environmental pollution. Mastitis is serious udder disease of different livestock resulting in poor animal health, deterioration of the quality of the produced milk and potential public health challenge in most cases. Although mastitis represents a nightmare for livestock industry, little is known on incidence, microbial community and molecular diversity of the causative agents in Ecuador. Bacterial mastitis is the main common form of the disease due to abundance and diversity of microbial agents in the farms' environment. Transmission includes contact of the teat with the infectious agent(s) either from surrounding environment or from animal to animal via milking practices or teat skin contamination. Hygienic level is a significant inductive factor, while transfer of infected animals between farms is a major disseminating factor. Management of the farms and hygienic conditions in the animal milieu are crucial factors in the occurrence rate of the disease. Treatment depends mainly on extensive use of antimicrobial agents. However, development of drug resistance in the microbial community and accumulation of toxic drug residuals in the food products are of major concern. Diversity in the microbial community causing mastitis is the paramount in any treatment and control regimens. Accurate identification of the causative agents and determination of sensitivity of isolated microbes to the used antimicrobial drugs effectively help treatment and control of the disease.

Molecular analyses of the collected isolates open new horizons to understand diversity of the mastitis microbial community, development of resistance and resistance genes, determination the source of infection and to track the infection. Therefore, the present study is delineated to spot analysis in details on molecular epidemiology of mastitis in beef cattle in El Oro Province, Ecuador, starting from the fall of 2013 for 2 consecutive years, and also accurate treatment and control of mastitis disease: (1) accurate identification of mastitis microbes through standard bacteriological procedures; (2) recommendation use of antimicrobial agents based on sensitivity/resistance to a wide panel of antimicrobial agents; (3) Determination of molecular diversity of mastitic bacteria and inferring their phylogenies; (4) statistical determination of factors interplayed on occurrence of mastitis; (5) elucidation of presence/absence of resistance genes in individual microbial strain/isolate; (6) determination the significance of recovered microbes as a public health concerns; (7) build up a risk factors analysis to help decision makers to forward strategic plans of veterinary and public health; (8) design a farm (herd) based management regimen to help control of the disease and increase farm sustainability and profitability. Based on the generated databases, appropriate management and control regimens of mastitis will be designed, not only in the El Oro Province but may be applicable on the national levels.

In this part mention clearly what will be the contribution of research knowledge in the respective area

In South American countries, bovine mastitis is very common, multifactorial disease with high impact on livestock industry. In these countries mastitis is caused by different pathogens, with no unequivocal description on the causative microbial agents. However, contagious bacteria including *Staphylococcus aureus* and *Streptococcuis agalactiae* are the most reported agents involved in bovine mastitis. Environmental *Streptococcuis uberis* is also repeatedly reported. Profound diversity is well documented in *Staph. aureus* from different South American countries, with indication of dominance of some clones in most infections. Horizontal gene transfer of some virulence associated genes is reported in some distinct staphylococcal strains involved in bovine mastitis, giving them a selective advantage when colonizing the mammary glands. Most isolated staphylococcal strains are potential toxigenic strains posing additional potential risk to public health. Furthermore, great diversity of genetic and virulence profiles are documented among bovine *Str. uberis* strains. However, coliform bacteria were the least reported agents. Conclusively, presence of these intramammary infections appeared to be associated with some management conditions, which highlighted the need to improve diagnosis and control measures of bovine mastitis

The output of this project will contribute in clear understanding the bacterial agents responsible for mastitis in beef cattle in El Oro Province. The generated results and knowledge will help the veterinarian to make the right decision on field treatments treatment policies, will help academics and scientific community to know in depth the genetic diversity and heterogeneity of mastitis microbes, will help farm owners to adopt cost-effective measurement conditions and cost-effective treatment policies to increase productivity, profitability and sustainability of the farms. Also, generated results and knowledge will help the policy makers to have the data and scientific recourses to build up the strategic plans and decisions on the animal health and management. Lab cooperation and workshops will help, knowledge, skill and experience transfer to the young generation of researchers/students.

The methodology used in the investigation. This section should demonstrate the feasibility of the research.

Study area and sampling protocols

Geographical and environmental conditions of El Oro Province will be obtained from local authorities. Also, distribution of the cattle farms (**herds on open pasture**), cattle breed, herd size, types of farms in terms of single or mixed animal species, milk quality, frequency of milk discarding, frequency of mastitis, longevity of the disease in the clinical/subclincal cases, costs of treatment of mastitis, frequency of recurrence, number of animals culled due to mastitis, number of employee in the farm/with the herd, profitability of the farm in terms of revenues and expenses ...etc, will be obtained by field questionnaires. A focused list of farms/herds to be sampled will be created. Milk samples from individual udder quarter or samples from udder exudates will be collected directly from cows with clinical or suspected subclinical mastitis under complete aseptic precautions. Then samples will be delivered to Bacteriological laboratory at Technical University of Machala. Samples will be subjected to somatic cells count (SCC) by direct or indirect methods, bacteriological and molecular analyses. Smeared sediment of centrifuged samples will be examined microscopically after staining with Lffler's methylene blue and Gram's stain. Electric conductivity test will be done to relate the levels of sodium and chloride ions in milk with milk quality. In some instance environmental samples will be taken to spot analysis on the potential source of infection. Results will be statistically analyzed to spot light on the socio-economic effect of mastitis on the livestock sector.

Bacterial identification

The identification of mastitis pathogens will be done according to the guidelines of the National Mastitis Council (1999). Samples will be streaked onto appropriate culture media including blood agar enriched with 5% defibrinated sheep blood cells, modified Edward's medium (or TKT agar), mannitol salt agar and MacConkey's agar. Culture-positive plate will be tentatively identified according to colony morphology, haemolytic characteristic at 24 and 48 h. Resulting bacterial colonies will be picked up and maintained on nutrient agar slopes for further identification. Smears from the recovered isolates will be stained their Gram's stain for microscopical examination.

Staphylococci: isolates initially characterized as *staphylococci* on blood agar plates, will be tested for haemolysis, pigment production, coagulase reaction (free and bound with Slidex Staph Plus test), catalase and production of β-lactamases.

Streptococci: Colonies yielding Gram-positive cocci on modified Edward's medium (or TKT agar), with catalase-negative and oxidase negative reaction will be subjected to CAMP test, sodium hippurate hydrolysis and Esculin. Carbohydrates fermentation will be conducted in peptone water containing lactose, maltose, mannitol, raffinose glycerol, salicin, sorbitol, sucrose and trehalose. Confirmation of *Str. agalactiae* isolates will be by Lancefield grouping with type B antisera (Slidex Strepto Kit, BioMerieux) and by a positive haemolytic result according to the CAMP test.

Coliform: Coliform bacteria will be isolated and identified to species level using standard bacteriological techniques, including colony morphology on MacConkey's agar and biochemical oxidase, catalase, methyle red test, H_2S production test, urease test, citrate utilization, Voges-proskauer, sugar fermentation and gelatin.

Serological grouping of isolates were performed with a commercial latex agglutination kit according to manufacturer's recommendation.

Antimicrobial sensitivity

The antimicrobial will determined using a Kirby– Bauer disc diffusion assay according to the standards and interpretive criteria described by CLSI (2008). The following antimicrobials will be selected for testing, based on:

(a) Licensing for mastitis treatment in cattle (penicillin, cefazolin, cefoperazone, pirlimycin, gentamicin, streptomycin, and amoxicillin–clavulanic acid).

(b) Use in human medicine (rifaximin, erythromycin, pirlimycin, vancomycin, chloramphenicol, tetracycline).

(c) Determine phenotypes for resistance determinants assumed to be located on genetic mobile elements (TET and ERY).

Briefly, a single colony of the recovered bacterial isolate will be inoculated into nutrient broth and incubated aerobically for 4-6 hours at 37°C. The culture will be then flooded on Muller-Hinton agar plate. Different antibiotic discs will then applied to the surface of agar and incubated aerobically at 37°C overnight. Zone diameter of growth inhibition will be measured with a caliper via plates' bottom.

Molecular typing

For emergences, commercial PCR kit for detection of mastitis pathogens will be used for rapid identification of mastitic pathogens by direct extraction of genomic DNA from bacterial pellet obtained from milk or udder exudates samples. Samples will be typed by sequencing analysis of hypervariable regions within the 16S rRNA gene for pathogen discovery and identification.

Regular, single colony from bacterial cultures will be used for DNA extraction. A standard PFGE technique will be performed for genotyping. Bacteria genomic DNA will be digested with the appropriate restriction enzymes. Grouping will be done based on the resulting banding pattern. Identification of clonal lineages obtained by PFGE will be determined by cluster analysis.

Based on the clusters generated from PFGE, selected isolates will be analyzed by MLST for microbial genetic variation. Primers and PCR conditions of multilocus gene set(s) of individual strain, cloning and sequence procedures will be determined according to the published data. Generated sequences will be edited and aligned with reference sequences in genebank according to the established procedures.

Virulence-associated genes will be detected to predict the impact and significance of the recovered bacteria. Virulence genes, PCR conditions procedures will be performed according published data for the isolated organisms. Established antimicrobial resistance genes such as Methicillin, macrolides, tetracycline, lincosamide, Pencillin resistance gene ...etc, will be detected by PCR according the published protocols. Phylogenesis inferred using sequenced data will be done based on available of relevance sequences in the genebank using relevant software.

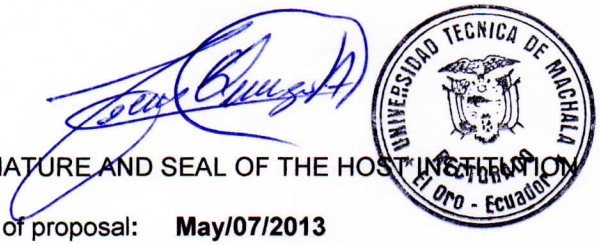
Expectations

In this section, please fill out the following table. Please do not modify the components and must meet mandatory components 1, 4 and 7. If any component is not applicable, please put N / A

COMPONENTS		SPECIFIC OBJECTIVES	OBJECTIVE RESULTS
1	RESEARCH	(1) Epidemiologic database Creation of epidemiologic database of incidence and associated factors such as hygiene, management factors and age and breed of the animal, nutritional status, seasonal variations ....etc. (2) Microbial database This database will be constructed on the bacteriological identification of mastitis-causing microorganisms. Sensitivity to	• Determination the incidence rates and the contributing factors will permit to decide the intervention measures, design a farm/herd based management regimen to control the disease. • Generation a microbial database both on the bacteriological and molecular levels will be beneficial both for regional and national use. • Precise databases will permit cost

		various antimicrobial agents, molecular diversity and heterogeneity, virulence factors and presence of genes associated resistance. (3) Risk Factors Based on the generated databases, environmental condition and management regimens, risk factors will be inferred after comprehensive statistical analyses.	effective treatment regimens, appropriate management regimens to control mastitis that will increase the productivity and financial sustainability of the farms/herd. • Five publications in peer reviewed journals are expected as scientific output of this project
2	SCIENTIFIC TRAINING IN THE RELEVANT AREA OF THEIR SPECIALTY (theoretical training)	Comprehensive descriptions will made available, in cooperation with the university and/or local authorities, to the stakeholders and veterinarians via farms/herd site visits, flyers and public conferences and workshops at the university premises.	Capacity building will help to plan the potential scenarios for appropriate intervention.
3	ADVICE ON THE DEVELOPMENT OF PUBLIC POLICIES	The compiled databases on the different sides of the disease including identification of microbial community, sensitivity to the antimicrobial agents, genetic diversity and virulence factors will be available to the policy makers	Based on the periodical reports and databases generated from this project, policy makers would be able to take the rational strategic decisions and plans related to animal health
4	TEACHING	Working in the labs. of Technical University of Machala give the opportunity to interaction with undergraduate and postgraduate students on lab courses on bacterial identification and different molecular techniques . Specific tailored courses could be designed according the need of the University.	This will permit in teaching of the new generations of researchers and graduates, knowledge transfer, different research skills and capacity building. Introducing a new courses according to the scope of the university
5	CONSULTING AND DESIGN GRADUATE PROGRAMS	The candidate is interested to participate in the design of graduate/postgraduate courses and programs in the light of more than 20 years experience with university teaching and aware with different curricula in the field	Participation of design graduate courses and program should add more experiences , scientific and practical values to the tailored courses and programs
6	MANAGEMENT NATIONAL AND INTERNATIONAL RESOURCES (administrative, human, economic, etc.).	**N/A**	**N/A**

7	STRATEGIC RELATIONSHIP BETWEEN INSTITUTIONS NATIONAL AND INTERNATIONAL	The candidate is interested to make cooperative channels with institutes in Japan, China and USA, rather than the home country Egypt. Each country has its own research and educational milieu and philosophy.	Cooperation with these different institutions will permit knowledge and technology transfer. Versatile knowledge from different sources in one pot which will enrich the outcome both on the scientific, educational and field levels. Also, it increases the international ties with Ecuadorian Institutions.



SIGNATURE AND SEAL OF THE HOST INSTITUTION

Date of proposal: **May/07/2013**