# 2 Project

2.1 Title (Maximum 60 characters)

**The role of complement and neutrophils in the dispersion of *Brucella* organisms and in the immune response, during experimental brucellosis**

2.2 Summary (Provide a layman's summary of your research proposal, including the aims and objectives 150 words)

Cells, named neutrophils that ingest, and serum proteins that kill, are in the first line of defense against brucellosis, a dangerous bacterial disease of domestic animals and humans caused by *Brucella* microbes. In spite of their function in the defense and in the immune response, very little is known of how neutrophils and serum proteins combat long lasting infections such as brucellosis. To understand this, we propose to investigate how *Brucella* resists the killing action of neutrophils and serum proteins and the regulatory events that occur during the immune response. Our objectives are: i) to determine the mechanisms by which *Brucella* interacts and resists the killing action of these elements ii) to determine the function of these elements in the modulation of host defenses iii) to develop a model for exploring the role of infected neutrophils as vehicles for the dispersion of *Brucella* in the different organs.

2.3 Abstract (Provide a scientific summary of your research proposal)

Neutrophils (PMNs) and complement proteins (C´) are in the first line of defense of innate immunity. The detection of various microbial structures by these elements influences the downstream activation events of the immune response. In spite of the significant role of complement and PMNs against most acute infections, the role of these elements of the innate immunity in bacterial chronic infections has remained unexplored for many years. In order to understand this, we propose to use the intracellular pathogen *Brucella abortus* as a model. Our studies have shown that *Brucella* resist the killing action of PMNs and C´, albeit these elements participate in the regulation of the adaptive immune response during brucellosis. Following this, our objectives are: i) To determine the mechanisms by which *Brucella* interacts and resists the bactericidal action of PMNs and C´; ii) to determine the role of C´ and PMNs in the modulation of adaptive immunity iii) to develop a model for exploring the role of PMNs as "Trojan horse" vehicles for the dispersion of *Brucella* to different organs.

**Please do not exceed this space**

# 3 Introduction

Provide a concise background to the project highlighting the question(s)/hypotheses to be addressed (Maximum 1 page)

*Brucella* organisms are responsible for a worldwide disease known as brucellosis. The disease is of public health, economic and ecological relevance since the bacterium affects humans as well as domestic and wild life mammals. All *Brucella* species are intracellular pathogens that invade monocytes (Mo), macrophages (Mø) dendritic cells (DCs) and placental trophoblasts. In pregnant host animals, such bovine, ovine, swine, caprine and canine, the reticuloendothelial system, placenta and mammary gland are the target organs, being abortion the most conspicuous symptom of brucellosis. In males are the testes. Humans acquire the bacterium through contact with infected animals or by ingestion of their products, mainly unpasteurized dairy foodstuffs. Brucellosis in humans is primarily characterized by undulant fever, arthralgia and myalgia, and if not treated, the bacterium may invade the heart and the central nervous system and cause death.

During the course of brucellosis Mo, Mø and DCs are recruited at the site of infection, and are the main effectors engaged by adaptive immunity. As in other bacterial diseases, PMNs and complement (C´) are the first elements of the innate immune system that encounter *Brucella* after invasion. However, in contrast to what has been observed with other bacteria pathgens, *Brucella* species are quite resistant to the microbicidal action mediated mediated by these two elements of innate immunity. Patients and animals infected with *Brucella* rarely display neutrophilia and the levels of C´ and other serum proteins remain unaltered. While *Brucella* actively invades and replicates within Mo, Mø and DC, the bacterium does not replicate in PMNs, but merely survives. Moreover, *Brucella* organisms induce low activation of PMNs and complement cascade and have evolved to resist the killing action of these elements of the innate immune system. This lack of activation in tune with the absence of early proinflammatory responses, lack of endotoxic symptoms and the almost total absence of coagulopathies in patients and animals infected with pathogenic *Brucella*.

In the course of our research, we have demonstrated that *Brucella* follows a stealthy strategy at the onset of infection. This property is related to the non-canonical structure and elements of the *Brucella* cell envelope that are not recognized by innate immune receptors as pathogen-associated molecular patterns, or PAMPs. During this process, *Brucella* avoids the recognition and activation of several elements of the innate immune response generating an immunological "window" that allows the bacterium to invade host cells of the reticuloendothelial system and replicate. In spite of this, PMNs play a significant role in the regulation of adaptive immunity during brucellosis, and *Brucella* infected PMNs died prematurely. The dead of *Brucella* infected PMNs may be linked to the absolute neutropenia observed in a proportion of brucellosis human patients, mainly during the chronic stages. Moreover, *Brucella* infected PMNs displaying death signals may be removed by cells of the reticuloendothelial system, such Mo, Mø and DC in a non- phlogistic manner.
Based on these findings we have put forward **the hypothesis that PMNs and C´ play a regulatory role in the immune response during brucellosis, and that *Brucella* infected PMNs serve as "Trojan horse" vehicles for the bacterial dispersion to different organs of the reticuloendothelial system.**
Following this, three questions remain:

i) what are the events associated with the interaction of PMNs and C´ components with *Brucella*?;
ii) which role play these elements of innate immunity during experimental brucellosis?, and:
iii) do infected PMNs serve as "Trojan horse" vehicles for *Brucella* dispersion to the different organs of the reticuloendothelial system, favoring the chronicity of brucellosis?

To achieve this we will focus in three specific aims:

1.- To determine the mechanisms by which *Brucella* interacts and resists the bactericidal action of PMNs and C.´
2.- To determine the role of C´ and PMNs in the modulation of adaptive immunity during experimental brucellosis.
3.- To develop a model for exploring the role of PMNs as "Trojan horse" vehicles for the dispersion of *Brucella* to different organs.

The results of this investigation will contribute to understand the strategy followed by furtive pathogens and their interaction with central components of the innate immune system. In addition, this investigation may lead to obtain relevant information for the development of therapeutic strategies based on the modulation of the innate immune response during early stages of infection by intracellular stealthy pathogens.

# 4 Research Project

4.1 Define specific research activities to be pursued during the project period and provide a comprehensive description of the techniques to be used and the advantages of the suggested methodological approach. Please include any selected relevant references. (Maximum 5 pages, including references)

**General strategy outline**

**Key objective:**

To unveil the events associated with the interaction of *Brucella abortus* with PMNs and C´, and determine the role that these elements play during in the regulation of the immune response and in the dispersion of the bacterium during experimental brucellosis.

To achieve this we will focus in three specific aims:

1.- To determine the mechanisms by which *Brucella* interacts and resists the bactericidal action of PMNs and C.´
2.- To determine the role of C´ and PMNs in the modulation of adaptive immunity during experimental brucellosis.
3.- To develop a model for exploring the role of PMNs as "Trojan horse" vehicles for the dispersion of *Brucella* to different organs.

**Research activities and techniques description**

**I.- Identification of *Brucella* putative proteins for the interaction of serum proteins and elements of innate immunity.**
It is known that the surface of bacterial pathogens display surface proteins such as adhesion molecules and serum binding proteins that interact with elements of the innate immune system. Following this, we will perform an *in silico* exploration of proteins that interact with complement, fibronectin, scavenger receptors, adhesion and serum resistance, dectins among several (particularly proteins involved in innate immune recognition) in the *Brucella* genome. For this we will explore sequences of already described proteins in other pathogenic bacteria which function is known. To achieve this we will use the bioinformatics tool BLAST (National Center for Biotechnology Information) and available bioinformatic databases (NCBI, KEGG, Kyoto Encyclopedia of Genes and Genomes). We will look for ortologue genes with a high degree of similarity among the *B. abortus* genomes**.**

**II.- Construction of *B. abortus* mutants related to recognition proteins.**
We already have a broad collection of *B. abortus* cell envelope mutants (e.g. LPS mutants, Omp mutants and lipid mutants). IN addition we will generate new B. abortus mutants in cell envelope elements identified according to the *in silico* comparative analysis described above ,that could be involved in the recognition of elements of the innate immune system. Mutants will be constructed in selected elements by site directed mutagenesis (a technique routinely used in our laboratory). Briefly, the gen of interest will be *knock out* following a PCR overlap technique. The introduction of this product in *B. abortus* cells will be by*Brucella* a recombination strategy.

**III.- Interaction of *Brucella* strains with PMNs and serum components from different animal hosts.**
It has been extensively demonstrated that *Brucella* organism are resistant to the bactericidal action of serum components and PMNs. In addition, we recently have shown that the interaction of serum components and PMNs of mice with *B. abortus* differs from that of humans. In order to understand this, first we will confront the collection of *Brucella* mutants with sera and PMNs from different animal hosts (human, cow, dog, sheep and mouse), and the killing and phagocytosis events recorded, accordingly. As controls other Gram negative bacteria (e.g *Salmonella*) will be used in the assays. Briefly, bacteria grown to mid log phase will be suspended in normal serum in the presence or absence of PMNs. As control another group of *B. abortus* will be suspended in inactivated/C´-deprived serum in the presence or absence of PMNs. The killing of bacteria by serum or PMNs will performed by incubating the assays at 37°C for 45-90 minutes. Aliquots of each reaction mixture will be plated onto Tryticase Soy Agar and incubated at 37°C for 3 days. Percent survival will be expressed as the number of colonies surviving at different times compared to the colony count incubated in inactivated/C´-deprived serum. Phagocytosis of bacteria will be recorded by fluorescent microscopy as performed previously. Cytokines produced in ex vivo will be recorded by ELISA in those assays involving interactions of *B. abortus* with PMNs from different animals. With these experiments, we envision to identify some of the *Brucella* cell envelope molecules involved in the interaction with serum components and PMNs from different animal hosts.

**IV.- Identification of C´ proteins and other serum proteins interacting with *Brucella***
The relationship between micro-organisms and the immune system is based on complex interactions. It is known that pathogenic *Brucella* induces low activation of the complement cascade and are resistant to complement killing. However specific interaction between serum proteins and the bacteria are not well understood. In preliminary data we have identified serum proteins (among them complement proteins) that interact with the outer membrane of *B. abortus* using the proteomic approach as the method for identification. For this purpose, different *B. abortus* as well as recognition mutants strains will be incubated with serum from different species (human, cow, dog, mice) for 45 minutes. Then cells will be washed and serum proteins eluted from the bacteria using glycine 0.2 M (pH 2.5). Protein eluates will be either directly subjected to HPLC and analyzed by

proteomic analysis or separated in SDS-PAGE in non-reducing conditions. Gel bands will be excised and treated for MS/MS analysis. Alternatively, they will subject to Western blotting analysis using a collection of antibodies against complement and other serum factors. The profile of interacting proteins will be determined by comparative analysis of the identified peptides with the available protein database. Results will be expressed as the qualitative and quantitative identification of serum proteins interacting with the bacteria. We expect to see difference in the protein profile according to the host serum form the various animal hosts. With these experiments we intend to identify the serum proteins different the various host animals that interact with the surface of Brucella. By using the different cell envelope *Brucella* mutants, we intend to extract information related to the *Brucella* cell envelope molecules interacting with the various serum components from different host animals, and hopefully relate them to a direct function.

**V.- The role of complement in *Brucella* infection and immune response: an *in vivo* model.**
The complement system is essential for innate immunity function and the first line of defense against microorganisms. Pathogenic *Brucella*e induce low activation of complement cascade and have evolved to resist the killing action of complement. In spite of this, the role of complement during brucellosis *in vivo* has not been explored. In our approach we intend to use a murine complement depleted model. Briefly, depletion of complement is accomplished using Cobra Venom Factor (CVF), a complement-activating protein from cobra venom. It is a structural and functional analog of complement component C3. CVF functionally resembles C3b, the activated form of C3. Like C3b, CVF binds factor B, which is subsequently cleaved by factor D to form the bimolecular complex CVF,Bb. CVF,Bb is a C3/C5 convertase that cleaves both complement components C3 and C5. The end product is complement consumption. To accomplish depletion a group of Balb/C mice are going to be chronically depleted of complement (every 3 days) by means of repeated intraperitoneal injections of CVF (ug of CVF per g of mice). The control group will be submitted to the same procedure but using PBS. C´depleted and control mice will be infected intraperitoneally with *B. abortus* (strain 2308) or C *B. canis* (bcanCR12) and mouse survival recorded. Bacterial counts in the spleen, liver, lymph nodes and bone marrow of mice will be determined 7 days after infection as well as proinflammatory cytokines (TNF-α, IL-6, IL-12, IL-10, IFN-γ). Histopathological findings will be also reported, spleens from infected and PBS-treated mice will be fixed in 10% neutral buffered formalin, processed and stained with hematoxylin and eosin or Giemsa stain as described elsewhere. In some cases spleen sections will be subjected to *Brucella* antigen detection. Results will be expressed as the comparison between de complement deficient mice (depleted and knock out) versus the corresponding control group. In bacterial counts, cytokine profile and histologic findings.

**VI.- Interaction of *Brucella* with PMNs at different time points of the infection.**
In previous works we have shown in the murine model that PMNs and C´ do not play a significant role at the onset of *Brucella* infection. However, we determined that PMNs play a significant function in regulating the Th1 response at early stages of experimental brucellosis. Following this, we intend to explore the role of PMNs during *Brucella* infection, once the adaptive immunity has fully developed. We will perform a set of two different but related experiments, using as background control mice infected with *B. abortus* 2308. In the first experimental group we will evaluate the *in vivo* response of neutrophils in terms of spleen bacterial load in the presence of anti-*Brucella* antibodies. For this, we will inject pre-immune serum before *Brucella* infection in mice and count bacteria in the different organs after 7-14-21 days after infection. In the second experimental model, *B. abortus* infected mice will be depleted of PMNs during the early stages of the adaptive immune response (eg 5-8 days after infection), by means of specific antibodies anti-PMN. In this second group bacterial counts will be performed at 14 days, 21 days and 30 days of *B. abortus* infection. In addition of counting bacteria in the different organs, we will determine in controls and experimental groups of mice the following: cytokines by ELISA, antibody isotype by ELISA, histopathological studies and immune cell populations (resting and activated) by flow cytometry. With this, we envision to detect which elements of the immune system are involved, once the adaptive immunity has developed in brucellosis. Although it is not within the scope of this proposal, the results obtained here will be essential for future investigations related to the understanding of the mechanistic cell interactions during the regulation of the immune response by PMNs and C¨.

**VII.- Development of a model for exploring the role of PMNs as "Trojan horse" vehicles for the dispersion of *Brucella* to different organs**.
We have proposed that PMNs, rather than destroying *Brucella* organisms, serve as "Trojan horse" vehicles for the dispersion of the bacterium to different organs of the reticuloendothelial system. In addition, we have proposed that this event occurs in non-phlogistic manner, avoiding excessive activation of Mo, Mø and DC, favoring the chronicity of the infection. In order to test this hypothesis, we intend to develop an experimental model in the mouse. Results from our laboratory have shown that in contrast to other mammals, murine naïve PMNs fail to engulf smooth *Brucella* and therefore unable to induce cell death in naïve mouse PMNs. However, in the presence of anti-*Brucella* antibodies, mouse PMNs successfully internalize this bacterium. Moreover, similar to what has been observed with human PMNs, antibody opsonized *B. abortus* is capable to induce the premature cell death of mouse PMNs. Accordingly, we will use antibody opsonized *Brucella* in the phagocytosis assays by mouse bone marrow PMNs. Since *Brucella* infected PMN´s display surface "eat me" signals, this infected PMN´s will be exposed to macrophages (cell culture and bone marrow derived) as well as dendritic cells. Then, phagocytosis of *B. abortus* infected PMNs will be recorded by confocal and electron microscopy. For *in vivo* experiments, *Brucella* infected mouse PMNs will injected intraperitoneally, intradermically of subcutaneously and then the phagocytosis by Mo, Mø and DC evaluated by intravital fluorescent microscopy and electron microscopy. In all cases the activation of phagocytic dells in the presence of infected Brucella PMNs will be evaluated by measuring the cytokine production and detecting activating markers in the surface of these cells. The goal is to determine if *Brucella* infected moue PMN´s are phagocytized in an anti-inflammatory or pro-inflammatory action and to evaluate if *Brucella* from this infected PMN´s are capable of replication within the phagocytes.

**Selected references**

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4.2 Time schedule (Specify work elements within the time frame of the project)

| Activities | First Year (bimonthly I) | | | | | | Second year (bimonthly I) | | | | | | Third year (bimonthly) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 | 30 | 32 | 34 | 36 |
| I.- Identification of *Brucella* putative proteins for the interaction of serum proteins and elements of innate immunity | | | | | | | | | | | | | | | | | | |
| II.- Construction of *B. abortus* mutants related to recognition proteins | | | | | | | | | | | | | | | | | | |
| III.- Interaction of *Brucella* strains with PMNs and serum components from different animal hosts | | | | | | | | | | | | | | | | | | |
| IV.-Identification of C´ proteins and other serum proteins interacting with *Brucella* | | | | | | | | | | | | | | | | | | |
| V.- The role of complement in *Brucella* infection and immune response: an in vivo model | | | | | | | | | | | | | | | | | | |
| VI.- Interaction of *Brucella* with PMNs at different time points of the infection | | | | | | | | | | | | | | | | | | |
| VII.- Development of a model for exploring the role of PMNs as "Trojan horse" vehicles for the dispersion of Brucella to different organs | | | | | | | | | | | | | | | | | | |
| VIII.- Preparation of the manuscripts, thesis, student´s training and publications | | | | | | | | | | | | | | | | | | |

4.3 Potential for training of young scientists & collaborations (Specify if training of young scientists and any travels are foreseen. Please indicate the potential for collaborations with ICGEB groups and/or other laboratories)

We have currently three graduate students in the lab enrolled in this project and their theses topics are related to the role of PMNs and complement in brucellosis *B. abortus*. Gabriela Gonzalez, Ricardo Mora and Cristina Gutiérrez are master students at the University of Costa Rica in the Microbiology Graduate Program. They have been trained in cytometry, proteomics (SDS-PAGE electrophoresis), biochemistry (Western blotting), cell infections, immunofluorescense techniques, construction of mutants and purification of proteins among other techniques. These graduates programs stimulate short term stays at foreign collaborative laboratories. Currently we have strong collaborations with the laboratory of Dr. Ignacio Moriyón, Universidad de

Navarra, Pamplona, Spain and Dr. Jean Pierre Gorvel, Immunology Center of Marseille-Luminy in Marseille, France. We intend to send our students to these laboratories in order to complete some experiments and enhance their education.

4.4 Facilities available in the Investigating Team's laboratory (Provide a detailed list of the infrastructure and equipment available and necessary for the proposed research)

FACILITIES AT THE LAB

- Bacterial growth and cell infections: Restricted access biosafety rooms to handle *B. abortus* (laminar hoods, bacterial incubators, spectrophotometer, centrifuges, immunofluorescense microscopy, cytometer
- Biochemistry and Protein purification: 1D and 2D (Multiphor, GE healthcare) apparatus, Chemidoc XRS for image capture (chemilluminiscense, silver and fluorescence staining), preparative super centrifuge, Ultra centrifuge, Ultrafiltration apparatus and accessories, chromatographic basic equipment (peristaltic pump, A215 reader, graphicator, fraction collector).
- Molecular biology: Agarose electrophoresis accessories, speed vac, gradient PCR machines

CORE FACILITIES AT THE UNIVERSITY

- Electron and confocal microscopy.
- MaldiTOF proteomic identification.
- Real Time PCR.
- DNA sequencing

4.5 Feasibility (Indicate the expertise of the PI and the assembled team that is relevant for performing the proposed research)

As a researcher I have close to 11 years of experience. I have done internships abroad, particularly in Immunology Center of Marseille-Luminy in France where I spent a year doing research. I have also experience in management tutorial undergraduate and graduate students. I speak and write English and have instrumental knowledge of French. For my research I have received the award for best paper presentation at the World Congress of Brucellosis in London in 2008, honorable mention in the presentation of my master's thesis and doctoral degrees, and recently the National Award for Science Clodomiro Picado Twight 2014.

Within the context of the project (in collaboration with national and foreign scientists), including Prof. Edgardo Moreno (National University, Costa Rica), Prof. Jean Pierre Gorvel (CIML, France), Prof. Ignacio Moriyón (UNAV, Spain) and Prof. Carlos Chacón (University of Costa Rica, Costa Rica) we have focused on defining and discover how *Brucella* behave as stealthy pathogen without being recognized by the innate immune system. This is a novel property, since bacteria are generally well recognized by cells of this ancient system. One of the core aspects of our research was the discovery that several surface structures of *Brucella* (which usually in other bacteria are recognized by the innate immune system) are modified. Therefore *Brucella* becomes "invisible" to the cells of the innate immune system and consequently not recognized.

Much of our research has focused on understanding the role of neutrophils during brucellosis and more recently the complement system. The key findings are that neutrophils are not only incapable of eliminating *Brucella*, but its intervention occurs unexpectedly at later times, and not as expected, during the early events of infection. We have proposed that this property allows *Brucella* to use neutrophils as "Trojan horses" to invade tissues and spread to other organs, which is the central aspect of this proposal that involves various systems, including the complement. Moreover, we found that neutrophils, besides being the first line of defense of the innate immune system, are also involved in the negative regulation of the adaptive immune system (long term), which proposes a new code in the control of immunity. Additionally, we discovered that *Brucella* induce the premature death of these cells, further eroding the participation of innate immunity in brucellosis.

Our research has contributed to understand the pathogenesis and immunity of brucellosis. This has opened a "path" of research to understand how some bacteria cause chronic diseases, such as tuberculosis or leprosy, are also able to evade the innate immune response. In this sense, our work transcends brucellosis and contributes to the general knowledge of the pathogenesis of bacteria that causes chronic disabling diseases, most of them of importance in public health, veterinary and wildlife.

## 5 Financial Contribution requested from ICGEB (all figures to be indicated in Euro)

Please read carefully the Budget Guidelines and provide annual breakdown, in Euro, of the requested funds together with a brief description of the foreseen expenditures.

| | 1st year | 2nd year | 3rd year | Total per budget category |
|---|---|---|---|---|
| Equipment[1] | 9000 | 4000 | | 13000 |
| Consumables & Training[2] | 15000 | 18000 | 21000 | 54000 |
| Travel[3] | | 3000 | 2000 | |
| Literature[4] | | | 2000 | |
| Sub total | 25000 | 25000 | 25000 | |

**TOTAL CONTRIBUTION REQUESTED FROM ICGEB**
**(The maximum annual contribution requested cannot exceed Euro 25,000)** **Euro 75000**

[1]Equipment (This budget category **must not exceed 30%** of the total grant requested/awarded – **please refer to the Budget Guidelines)**

1) Ultra freezer
2) $CO_2$ Incubator
3) Shaking incubators
4) Sonicator
5) Stand desiccator

[2]Consumables & Training – **please refer to the Budget Guidelines**

- Lab reagents for bacterial culture and molecular biology: culture media, buffers, antibodies, enzymes, cell death reagents, chromatographic matrices, fluorochromes, cytokine determination kits, cytometry supplies.
- General consumables: Plastic recipients, pipette tips, pippettes, disposables, Nitrogen, $CO_2$ tanks, Water purification accessories.
- Short term training visit of graduate students to collaborators.

[3]Travel
This budget category **must not exceed 10%** of the total grant requested/awarded – **please refer to the Budget Guidelines**

1) Participation at scientific meetings for dissemination of results (Brucellosis International Conference)
2) Visit to collaborative laboratories
3)

[4]Literature This budget category **must not exceed 5%** of the total grant requested/awarded – **please refer to the Budget Guidelines**

Publications costs

Journal subscription

## 6 Proposed Referees

(Provide the name and full coordinates of a maximum of 3 referees who would be willing to review your proposal. Please note that the ICGEB will have the sole responsibility in deciding whether or not a proposal will be submitted for evaluation to the referee(s) listed below)

Referee No. 1

| | |
|---|---|
| Surname | Tsolis |
| First Name | Renee |
| Institute address | University of California, Davis<br>One Shields Avenue, Genome and Biomedical Sciences Facility<br>Room 5519 Davis, California 95616 |
| Tel: | (530) 754-8497 |
| Fax: | (530) 754-7240 |
| E-mail: | rmtsolis@ucdavis.edu |

Referee No. 2

| | |
|---|---|
| Surname | Roop |
| First Name | Martin |
| Institute address | East Carolina University<br>The Brody School of Medicine<br>Department of Microbiology and Immunology |
| Tel: | (252) 744-1357 |
| Fax: | Fax: (252) 744-3535 |
| E-mail: | roopr@ecu.edu |

Referee No. 3

| | |
|---|---|
| Surname | |
| First Name | |
| Institute address | |
| Tel: | |
| Fax: | |
| E-mail: | |

## 7 Feedback

(Please indicate, selecting only one choice, how you found out about the Collaborative Research Programme (CRP) – ICGEB Research Grant Programme)

| | |
|---|---|
| ICGEB Website | ☐ |
| Social networks | ☐ |
| ICGEB Liaison Officer | ☐ |
| Your University/Institute | ☐ |
| A colleague | X |
| Other (please specify) | ☐ |

# ANNEX A - ICGEB Member States

**eligible to apply for funding under the CRP – ICGEB Research Grant Programme**

*For name of Liaison Officers and full contact details see:*
http://www.icgeb.org/member-states.html

AFGHANISTAN
ALGERIA
ARGENTINA
BANGLADESH
BHUTAN
BOSNIA AND HERZEGOVINA
BRAZIL
BULGARIA
BURKINA FASO
BURUNDI
CAMEROON
CHILE
CHINA
COLOMBIA
COSTA RICA
CÔTE D'IVOIRE
CROATIA
CUBA
ECUADOR
EGYPT
ERITREA
FYR MACEDONIA
HUNGARY
INDIA
IRAN (ISLAMIC REPUBLIC OF)
IRAQ
JORDAN
KENYA
KUWAIT
KYRGYZSTAN
LIBERIA
LIBYA
MALAYSIA
MAURITIUS
MEXICO
MONTENEGRO
MOROCCO
NAMIBIA
NIGERIA
PAKISTAN
PANAMA
PERU
POLAND
QATAR
ROMANIA
RUSSIAN FEDERATION
SAUDI ARABIA
SENEGAL
SERBIA
SLOVAKIA
SLOVENIA
SOUTH AFRICA
SRI LANKA
SUDAN
SYRIAN ARAB REPUBLIC
TRINIDAD AND TOBAGO
TUNISIA
TURKEY
UNITED ARAB EMIRATES
UNITED REPUBLIC OF TANZANIA
URUGUAY
VENEZUELA (BOLIVARIAN REPUBLIC OF)
VIET NAM

# CRP - ICGEB Research Grant Application Form 2016

Check List for Principal Investigator

Have you completed all the sections of this application form in English? ☐

Have you signed Form A? ☐

Has the Legal Representative of your Institute signed Form A1? ☐

Have you completed section 5 (e.g., Financial contribution requested from ICGEB) according to the Budget Guidelines? ☐

Is the budget expressed in Euro? ☐

Submit your proposal by e-mail (as a pdf attachment) **BOTH to**: ☐

1) the Liaison Officer of your country (refer to Annex A for the list of eligible countries and for full contact details)
2) the CRP-ICGEB Research Grant office (crp@icgeb.org)

**For ICGEB Liaison Officers**

**Please note that incomplete applications will not be processed**