**Intervention study to prevent bacterial hospital-acquired infections by oral treatment with IgY antibodies from eggs.**

1. **General aims**
   This project is aimed at determining if and how IgY antibodies from eggs can be used to immunize humans to prevent infections with multi-resistant ESBL-producing Gram-negative bacteria such as Klebsiella and E. coli. This will be studied in two subprojects.
   --An in vitro analysis of the effects of IgY on bacterial adhesion, growth and survival.
   --A clinical treatment study at the University Hospital in Uppsala to determine if IgY can prevent colonization and transmission of multi-resistant ESBL Klebsiella.

2. **Project aims and Results**
   **Project aims:**
   **An in vitro analysis of the effects of IgY on bacterial adhesion, growth and survival.**
   Effect of IgY on survival and growth.
   At present it is unclear by which mechanism(s) IgY affects bacterial colonization ability. One possibility is that adhesion is impaired or alternatively growth is reduced. We will determine how bacterial biofilm formation, binding to eukaryotic cells and growth is influenced by increasing IgY levels.
   Identification of the most important surface antigens and their effect on adhesion.
   One possible effect of IgY is that it blocks important surface structures such as a fimbriae and/or capsule that are important for cell adhesion. We will use mutants with defects in production of these components to determine how IgY influences cell binding. This approach will allow us to determine if and how the different surface structures are important for cell adhesion and if IgY can inhibit binding.

   **A clinical treatment study at the University Hospital in Uppsala to determine if IgY can prevent colonization and transmission of multi-resistant ESBL Klebsiella.**
   Hens will be vaccinated with killed ESBL-producing Klebsiella isolated from patients at the Uppsala University Hospital and IgY will be isolated. Patients will be treated with IgY solution 3 times /day for 14 days in a blind randomized study.

**Results:**
**A. Binding of IgY to Klebsiella pneumoniae.** IgY antibodies against ESBL-producing Klebsiella were developed and their activity was investigated with several methods.

*Biofilm assay:* Biofilm formation is an important virulence factor. We investigated if IgY could prevent biofilm formation, which would indicate binding of IgY to Klebsiella and possibly a therapeutic effect. This was examined in a micro-well plate assay. Briefly, Klebsiella were cultivated and transferred to micro-well plates. To some wells IgY was added. Thereafter the bacteria were allowed to form biofilm during different time points. Finally, the biofilm was stained with crystal violet and the absorbance read as a measurement of biofilm formation. In spite of much effort to optimize the assay, we had problems with large intra-assay variations and have not been able to draw any conclusions so far using this assay system.

*Hemagglutination assay:* Klebsiella expressing type 3 fimbriae agglutinates ox erythrocytes treated with tannic acid. We aimed to study if IgY could prevent hemagglutination, which would show that the antibodies bind type 3 fimbriae. Similar to the biofilm assay this assay system showed large intr-assay variation.
**ELISA:** ELISA experiments were performed to determine if IgY bound to different strains of Klebsiella. It was found that specific IgY bound to all strains tested, whereas there was no reaction when control IgY was added to the wells. Thus, the IgY produced were specific for the Klebsiella used for immunization.

**Flow cytometry:** By flow cytometry analysis the relative amount of IgY bound to Klebsiella strains was determined. IgY bound to most strains with some variation in extent of binding. When binding was detected, the specific IgY bound more than control antibodies.

**Immunostaining:** The binding of IgY to Klebsiella was visualized by immunostaining. First, IgY was incubated with Klebsiella to allow binding and then with a fluorescent antibody against IgY. This assay showed that the specific IgY antibodies bound specifically to Klebsiella.

**B. Identification of antigenic proteins.** It was of interest to identify the Klebsiella proteins antigenic for IgY to potentially find a mechanism by which IgY could prevent gastrointestinal colonization with ESBL-producing Klebsiella. The first step to identify proteins was to purify Klebsiella proteins. Then, proteins from individual strains were separated by 2D gel electrophoresis. The gels were either stained with colloidal blue to see all proteins or western blot was performed to visualize only the proteins antigenic for IgY. The protein patterns on 2D gels and western blots were similar between strains. When the blots were incubated with specific IgY several spots appeared. Some spots appeared with unspecific IgY as well, but not the same ones as with specific antibodies. The gel chosen for protein analysis was of a strain (DA11912) with a western blot most alike that of the other strains. The gel and blot was visually matched and 13 proteins were chosen for identification. All proteins could be identified with mass spectrometry analysis. Most of them were localized in the membrane and they had functions in energy metabolism and transport.

**C. Measurements of growth rates.** In order to determine if IgY has an effect on growth rates of Klebsiella a number of strains were grown in the presence or absence of specific or unspecific IgY. The exponential growth rates were measured in a Bioscreen reader that records the absorbance every fourth minute. It was found that IgY reduces the growth rates of clinical isolates carrying the same ESBL-plasmid as those used for immunization to develop the antibodies. Unspecific IgY had no effect on these strains. This result is very promising since it shows that the IgY antibodies can specifically and significantly reduce the growth rate of the relevant strains.

**D. IgY antibodies.** Hens have been immunized with the relevant Klebsiella strains and IgY has been purified and is ready to be used for the clinical trial.

**E. PhD students.** Two graduate students Elin Nilsson and Thomas Tängden have been working on characterization of the IgY antibodies and ESBL Klebsiella as well as the clinical study aiming to use IgY for treatment to prevent colonization and/or transmission of ESBL Klebsiella. Elin Nilsson will present her PhD thesis in the spring of 2009 and her work has been instrumental in defining the IgY antibodies and their effect on the bacteria.

4. **Deviations from project plan**

The project has been following the initial project plan but since the experiments to define better the effect of the IgY antibodies on bacterial growth and adhesion took much longer than expected, we have still not begun the clinical study. These additional experiments that we have performed during 2008 were requested by the Medical Products Agency (Läkemedelsverket) before we would start the trial. With the above results in hand we will now proceed to obtain the
final ethical permit and start the actual trial. The use of IgY to prevent Pseudomonas infections in cystic fibrosis patients has received orphan drug status from EMEA (European Medicines Agency), which will most likely facilitate the execution of the present study.

5. Dissemination and use of results
The generated results have been and will be published in scientific journals and used as background knowledge and for the analysis of the outcome of the next-coming clinical trial. If this trial is successful the generated results will be very useful in determining why and how the IgY antibodies could affect colonization and/or transmission.

6. Collaborations
This project has been a highly collaborative project and has involved all of the original applicants (Prof. Dan Andersson, Prof. Otto Cars, Prof. Göran Friman, Prof. Hans Kollberg, Prof. Anders Larsson, Doc. Åsa Melhus, PhD student Elin Nilsson, PhD student Thomas Tängdén) as well as some other researchers. AFA’s funding has therefore not only allowed us to perform these studies but also generated long-term and ongoing scientific collaborations between researchers from quite different areas.

7. Publications
References 1-6 provide background knowledge for these studies and will be part of Elin Nilsson’s PhD thesis. Reference 7 is directly relevant to this proposal.