JESSICA RUGGERI: SHORT TERM VISIT REPORT 2015

BACKGROUND:

My research activity was focused on vaccine development against *Salmonella* Typhimurium and its Monophasic variant in pigs.

Salmonella Typhimurium represents a main economic problem in piggeries, due to diarrhea and weight loss of piglets, furthermore, antibiotic treatments are expensive and number of multi-resistant strains is increasing. Moreover, *S.* Typhimurium is a Health Public Issue and pigs and their products represent the main source of infection for humans. It is well recognized that chronically infected pigs introduce bacteria throughout the slaughterhouse and the food chain. EFSA reports indicate that the percentage of salmonellosis in humans is reduced during the last years, but the number of human cases of salmonellosis caused by *S.* Typhimurium and its Monophasic variant is increasing, and pork represents the main source of infection with these serotypes. This aspect is related to the introduction of mandatory vaccination in avian farms and, for this reason, the number of salmonellosis cases in humans due to the consumption of chicken meat contaminated with *S.* Enteritidis is reduced.

The control of infection in farms occurs through antibiotics treatments, bio-safety procedures and administration of alternative products, such as pro-biotics and acidifiers. However, these procedures are not sufficient in endemic farms and in endemic country, like Italy. For this reason, vaccination represents the main tool to control infection. Unfortunately, an efficacious and safe vaccine to control infection in piggeries is not available, yet. At this moment, EU community asks to membership states to control infection only in broilers and laying hens, but we cannot exclude that also for pigs vaccination will be mandatory.

In recent years, my research group has tested an attenuated vaccine of *S.* Typhimurium in experimental conditions and an inactivated *S.* Typhimurium Monophasic variant in field conditions. Results were excellent, but both vaccines have disadvantages. It is in common opinion of researchers that attenuated vaccines are less safe but more immunogenic than inactivated ones against some intracellular facultative bacteria as *Salmonella* and extraintestinal *E.coli*.

The aim of my visit in the Veterinary School of Nottingham University was to develop a new synthetic medium that will allow bacteria to grow in particular conditions, which mimic macrophage environment, favouring expression of virulence factors. The broth culture would then be inactivated and used as a more immunogenic inactivated vaccine.

ACTIVITY:

The first step of my visit was to organize next work. I had several meetings with my supervisor to define and organize my project. I had 3 courses for the laboratory induction, related to safe procedures and use of laboratory tools (cabinet, centrifuge, incubator, etc.) and I looked laboratory technicians and PhD students working to understand methods and techniques useful for my laboratory project. In this context, I had a complete trial in molecular biotechnology to understand microarray method, in particular, the description of the machine and the applied technique to my project. I assisted biologists to design array and the procedure consisted in analyzing complete genome of reference strain and design probe for our analysis, finally, 517 genes were checked in this project.

The second step of the visit was the laboratory activity, organized in: strains cultivation, RNA extraction and hybridization for microarray analysis, data collection and manipulation, formulation of a new synthetic medium, bacteria growth in new synthetic medium, new microarray analysis of the strains in new medium to check genes expression.

Strain cultivation:

S. Typhimurium and *E.coli* strains (Barrow's collection) were grown in macrophages cell lines and in Nutrient broth (Oxoid), as a control for the microarray analysis. Cells were infected with a 20 fold dilution of NB culture for one h in 5% CO2. After the incubation, inoculum was replaced with cells medium (RPMI, Gibco) containing antibiotics. After 8 h, cells were lysed for bacterial enumeration and perform microarray analysis. Nutrient broth (Oxoid) was grown overnight and 20 fold dilution were performed to infect macrophages, to count bacteria and to perform microarray analysis.

RNA extraction and hybridization:

The RNA extraction from cell cultures and 20 fold dilution of Nutrient broth (Oxoid) cultures happened 8 h after infection. For the first protocol, cells were lysed and centrifuged to collect bacteria grown inside. The pellet of bacteria was treated with lysozyme and proteinase to lyse cells wall and RNA was collected using RNeasy MINI KIT (Qiagen). Similar protocol was applied to broth culture in Nutrient Broth (Oxoid). The most important aspect was the number of bacteria in macrophages and Nutrient broth (Oxoid). The number of bacteria must to be very similar in both cultures to compare genes expression. Quantity and quality of RNA was checked by a Byoanalizer. RNA was amplified and labelled with fluorescent dies (Cy3 for control RNA and Cy5 for bacterial RNA from macrophages culture).

Microarray

This technique allowed simultaneous analysis of expression of hundreds of genes. It was important being careful because slight variations, in conservation and processing of samples, could determine

significant changes in genes expression. The fluorescence intensity was correlated to the amount of mRNA, and so the level of gene expression, presented in the original sample.

*S.*Typhimurium and *E.coli* genome was checked into Unigene and GenBank platform. We designed some probes of DNA (length 50-100 bp), representative of each gene, by PCR and using specific primers. The probes for *S.* Typhimurium are 746 and for *E.coli* were 517, some of these were already designed because used in common research projects. These probes were printed (spotted) in a microtiter plate by a specific instrument (Agilent eArray system).

The second step was RNA extraction from cell and broth cultures. We counted bacteria in cells and broth. We did several dilution of broth to obtain the same amount of bacteria present in cells. RNA was extracted through RNeasy Kit from cell and broth cultures. The sample was checked by a spectrophotometer to analyze purification and amount of RNA in each sample.

Extracted RNA was converted back in cDNA. The cDNAs from cells and broth cultures were labelled with two different fluorescent substances, Cy3 for cell sample and Cy5 for broth culture. Both of cDNAs were spotted together to hybridize with probe for 24 hours (competitive binding with probe). After time, plate was washed and analyzed by a scanner to read fluorescence of two samples. Results could be: only fluorescence of Cy3, only fluorescence of Cy5, no both fluorescence or both fluorescence, which intensity was determined by this ratio Cy3 fluorescence/Cy5 fluorescence.

Fluorescence results were normalized and 10 fold difference between Cy3 and Cy5 intensity was considered significant and gene up-regulated.

Data collection and Manipulation:

Data collected for 746 (*S.* Typhimurium) and 517 genes (*E.coli*) were: length, location, normalized fluorescent and fold versus control expression. Genes of *S.* Typhimurium and *E.coli* cultivated in macrophages were considered up-regulated when the difference of fluorescence was higher of 10 fold in comparison to fluorescence of genes from *S.* Typhimurium and *E.coli* grown in standard medium. The number of up-regulated genes was 319 in *S.* Typhimurium and 261 in *E.coli*. This meant that 319 and 261 genes were expressed by *S.* Typhimurium and *E.coli*, respectively, if they grew in macrophages, but not in standard medium.

The most demanding activity was to understand function of each up-regulated gene. The role of genes and of product, for which up-regulated gene encoded, was verified into Uniprot and Unigene platform. Then, genes were classified in relation to their function in different fold: electron and ion transport, homeostasis regulator, pH maintenance, regulator of key function, uptake of amino-acids and carbon sources. Analysis of these genes, related to analysis of standard media composition (Luria Bertani, Nutrient Broth, RPMI and etc), allowed us to verify what kind of corrections might be done in new synthetic medium composition.

Formulation of a new synthetic medium:

The analysis of gene expression allowed us to formulate a new synthetic medium. The new medium for *S.* Typhimurium and *E.coli* might to have a value of pH near 5. Low pH concentration was responsible for induction and expression of virulence factors, such as SPI 2 in *S.* Typhimurium and curli fimbriae in *E.coli*. Adding Tris-HCl permitted to maintain low pH values. An important related aspect was to avoid high concentration of carbon source whose metabolism produced acetic acid resulting in a further reduction of pH values. Excessive low pH values resulted in a cessation

of bacteria growth, consequently it was important to balance ingredients concentration and oxygenation.

The best osmolarity range was 0.03-1.0. These values permitted bacteria to grow and replicate. Lower values were responsible for excessive concentration of water into the cells, whilst high values were responsible for turgor loss and the concentration of ions and macromolecules could inhibit DNA replication. Some metal (K^+) and amine (Proline and Betaine) were continuously transported inside and outside cytoplasm to preserve cells function. These ingredients were added to the new synthetic medium.

Analysis of metal transport underlined the importance to add some metals as Magnesium, Sodium and Calcium to the medium.

In relation to carbon source, *Enterobacteriaceae* preferred C6 sugars as glucose, galactose and glycerol. All genes, involved in Pentose Pathway, were down-regulated. Glycerol was preferred in comparison to glucose and galactose. High concentrations of these sugars determined an excessive production of acetic acid which caused a further reduction of pH which could stop the growth of bacteria.

Amino acids played an important role in protein synthesis, furthermore they were important carbon and nitrogen source. Genes involved in some amino acid metabolism (*livJ*) were up-regulated, indicating that was important inserting them in the medium.

The last step was to establish the concentration of ingredients. For each of them, we decided different concentration values, in relation to indications from the literature and concentration of common standard media. The mg/l concentration was obtained by multiplying Molecular Weight and Molarity of each ingredient. The osmolarity of the new medium was measured by a freezing

point osmometer (OSMOMAT 030, Gonotec). Different attempts were performed to obtain the correct osmolarity value. Media was prepared and sterilized for use.

Bacteria growth in new medium:

After sterilization of the new media, a loopful of *S.*Typhimurium and *E.coli*, grown on selective agars, was re-suspended in 100 ml of the new media and incubated at 37°C (*S.* Typhimurium) or 37°C and 41° C (*E.coli*) in aerobic condition overnight. The turbidity of the media revealed the growth of bacteria and an aliquot was plated onto selective agars to check absence of contaminants. Broth cultures were counted and diluted to obtain the same condition of growth in macrophages. Then, they were centrifuged, bacteria wall were lysed and RNA extracted and collected with the same protocol described above. A new microarray analysis was performed to check gene expression of bacteria grown in the new medium. Collected data were compared to gene expression of bacteria grown in macrophages. Different concentration of ingredients determined slight differences in gene expression.

In conclusion, the composition most closest to macrophages environment was selected to produce vaccine. The strain was diluted in this media and grown in a bio-fermenter overnight, then inactivated with formalin 10 %.

My visit and the laboratory activities finished but, probably, the collaboration will continue to test the efficacy of these new vaccines.

The efficacy of the vaccines must be tested in animals. Good results were obtained by *in vitro* tests but the complex interaction between pathogen and host determined the necessity to check the vaccine efficacy *in vivo*. Furthermore, we know that attenuated vaccines are more immunogenic than inactivated ones because of their capability to stimulate cellular and humoral immune systems.

Inactivated vaccine principally determines antibodies production which is less protective against facultative intracellular bacteria such as *S.* Typhimurium and some serotypes of *E.coli.*

Experimental trials in animals will allow us to understand if these inactivated vaccines, characterized by containing bacteria which express more virulence factors, will be capable to stimulate host immunity, principally cellular immune system, as attenuated or live strains.

Data will be available for a peer-review article.