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Epidemiology of Non-Typhoidal *Salmonella* (Nts) in Humans and Animals in the Gambia and Senegal

Michel Dione

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Members of the Examination Committee

Prof. A. Gadisseur (UA),

Prof. J-P. Bogers (UA),

Prof. P. Bols (UA),

Prof. L. De Zutter (UGent),

Dr H. Imberechts (CODA, Brussels).

Results

PHD.in Medical Sciences

Summary

Non-Typhoidal *Salmonella* (NTS) species are important food-borne pathogens. Although acute gastroenteritis is the most common clinical symptom, complications can occur resulting in bacteraemia with or without focal infections. Food products, especially food of animal origin such as poultry are associated with the transmission to humans. In Africa, NTS are among the most common cause of bloodstream infections in children younger than 5 years. Epidemiological data on NTS are lacking in Africa both for human and animal infections. Therefore, a study providing a better understanding of the factors that lead to the emergence of NTS is a prerequisite for the design of improved intervention strategies to control these pathogens. The aim of this thesis was to study the epidemiology of NTS pathogens in humans and animals in The Gambia and Senegal.

Chapter 1 reviews the current status of knowledge on NTS infections in Africa with focus on The Gambia and Senegal. It also provides the background against which these studies were conducted.

Chapter 2 describes the prevalence of NTS along the poultry production chain in Casamance, Senegal. Fifty seven randomly selected broiler farms, 42 street restaurants and 285 chicken carcasses were studied. The following farm prevalences were reported: 35.1, 38.6 and 29.8% in chicken faeces, on carcass skin, and in muscles, respectively. NTS were found in chicken meat servings of 14.3% of the 42 street restaurants and in 40.4% of the 285 chicken carcasses examined. The most prevalent serotypes among the eighteen identified were *Salmonella* Brancaster (57.9%), *Salmonella* Goelzau (10.7%), *Salmonella* Kentucky (8.4%), and *Salmonella* Hadar (7.3%). The following serotypes were for the first time identified in Senegal: *Salmonella* Bandia, *Salmonella* Bessi, *Salmonella* Brunei, *Salmonella* Hull, *Salmonella* Istanbul, *Salmonella* Javiana, *Salmonella* Magherafelt, *Salmonella* Molade, *Salmonella* oxford, *Salmonella* Poona, *Salmonella* Rubislaw, *Salmonella* Tamale, *Salmonella* Zanzibar and *Salmonella* Goelzau. The prevalence of NTS on skin and in muscle was significantly associated with the detection of *Salmonella* in feces ($P \leq 0.001$). The high levels of contamination of skin and muscle can be attributed to poor hygiene at the farm level and the non-hygienic handling of chicken carcass meat during and after slaughtering. This conclusion is supported by the fact that some serotypes are present both on the farm (as found in feces) and in carcasses (on skin and meat). Food can also become contaminated through environmental contact, because hygienic measures applied in the restaurants are poor. A large proportion of our

isolates (77.7%) were resistant to two or more antibiotics commonly used in Senegalese veterinary practices and in human medicine (trimethoprim-sulfamethoxazole, tetracycline, trimethoprim, streptomycin, sulfonamides, and spectinomycin). The high prevalence of *Salmonella* in broilers in Casamance and the level of antibiotic resistance are of concern and constitute a real threat to public health.

Chapter 3 reports on the molecular characterization of 261 NTS serotypes isolated in poultry and poultry products in Senegal using consecutively RAPD and MLST. These techniques have provided details on the genetic diversity among the serotypes. Twenty distinct profiles were generated by the RAPD assay, the latter corresponding to the eighteen strains obtained after serotyping. *Salmonella* Kentucky showed two distinct profiles; this distinction was later confirmed by MLST. The MLST assay revealed genetic diversity resulting in 19 clones of which 16 were new and have never been reported anywhere in the world. The three known clones, namely *Salmonella* Kentucky ST198 previously reported in Senegal, *Salmonella* Agona ST13 and *Salmonella* Istanbul ST33 were isolated in many countries from both human and animal sources. This shows that these clones are geographically widely distributed and are circulating in a wide range of hosts. However, one new clone of multi-resistant *Salmonella* Kentucky was found. This study provided us with new insights into the genetic diversity on NTS in Senegal. Molecular tools remain essential to study the epidemiology of NTS by tracking the sources of infection and/or contamination. These same techniques were used to study the animal to human transmission in The Gambia in the next chapter.

In **chapter 4**, eight diarrheic children with confirmed salmonellosis and 6 healthy carriers were traced back to their compounds and *Salmonella* identified from the domestic animals (poultry, sheep and goat) living in close contact in the same compound. The most common serotypes identified were *Salmonella* Colindale in humans (21.42%) and *Salmonella* Poona in animals (14.28%). Among the animals, poultry carried the highest proportion of *Salmonella* (66.7%). In fact, poultry are considered as the most common asymptomatic carriers of *Salmonella*. However, serotypes in humans were different from those in animals except in one case where *Salmonella* Moulaine was simultaneously found in chicken and a diarrheic child but in different compounds. After proceeding MLST on all isolates, we found that those two *Salmonella* Moulaine were distinct but genetically very close because they differed at only one locus *sucA*. The similarity matrix of the strains revealed close genetic relatedness among *Salmonella* serotypes. There was at least 80% similarity and the majority varied between 98% and 100%. This showed the stability of *Salmonella* clones which are not subject to high genetic variability. There was therefore no indication of clonal groups which are adapted to a specific host because the genetic tree did reveal that all lineages contained isolates of mixed origin (human and animal). The association between salmonellosis and other diseases, most often malaria, in our study shows the role of opportunistic infections and malaria in NTS infections. Almost all serotypes were susceptible to all antibiotics tested. This is due to the fact that antibiotics are not yet commonly used by of the rural population in The Gambia for treatment of NTS infections as well in humans as in the animal production system. Our results do not support the hypothesis that humans and animals in close contact in the same household carry genotypically similar *Salmonella* serotypes. Nevertheless these findings have stirred up the problem of the transmission of NTS in Africa and have highlighted the poultry population as playing a pivotal role of healthy carriers in the epidemiology of NTS. Based on this study, we suggest other areas to be investigated such as the environment and human-to-human transmission.

Little is known on the molecular epidemiology of NTS particularly with respect to their virulence genes. Therefore, to assess their occurrence and contribution to disease in humans and animals in The Gambia and Senegal, we screened all serotypes isolated from humans, animals and food in both countries (**chapter 5**). A total number of 185 NTS was tested by PCR for the presence of 12 virulence genes. Among these genes, 10 belong to the five described *Salmonella* Pathogenicity islands thought to be implicated in *Salmonella* pathogenesis; and the other two genes are carried by plasmids. All genes were present at a level of more than 70% except *sopE* and *pefA* which were observed in 33% and 44% of the isolates, respectively. The most prevalent gene was *invA* (95.5%) which is an invasion gene conserved within the *Salmonella* genus. It has been widely used to diagnose *Salmonella* in humans and animals. However, the *sopE* gene associated with outbreaks in human and animals was present in all serotypes isolated in humans with diarrhoea except one. Interestingly, *Salmonella* Istanbul and *Salmonella* Javiana isolated from chicken serving restaurants carried all the virulence genes of the five pathogenicity islands. There was a significant association between some virulence genes (*sopB*, *sopE* and *pipD*) and resistance to certain antibiotics namely amoxicillin, ticarcillin, trimethoprim plus sulfamethoxazole, tetracycline, trimethoprim, spectinomycin, streptomycin, sulfonamides and nitrofurantoin. This association shows that resistance might increase the virulence of NTS during infection. These findings showed that all strains of *Salmonella* isolated from humans and animals are potentially pathogenic. This is very worrying because the most virulent serotypes were also the most frequently detected in food and in animals, and caused diarrhoea in immuno-compromised children. Furthermore, virulence and resistance are intrinsically linked and the presence of the virulence genes *sopB* and *pefA* is likely to increase the resistance to a series of antibiotics (trimethoprim plus sulfamethoxazole, tetracycline, trimethoprim and sulfonamides).

TSOL18 Vaccine Antigen of *Taenia solium*: Development of Monoclonal Antibodies and Field Testing of the Vaccine in Cameroon

Emmanuel Assana

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Promoter

Prof. Dr. P. Dorny
Faculty of Veterinary Medicine, UGent
Institute of Tropical Medicine, Antwerp
Prof. Dr. S. Geerts
Institute of Tropical Medicine, Antwerp

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Members of the Examination Committee

Prof. M. Lightowlers (Univ Melbourne, Australia), Copromoter
Prof A. Zoli (Univ Dschang, Cameroon), Copromoter
Prof J. Vercruyse (UGent),
Prof E. Cox (UGent),
Dr. Sarah Gabriel (ITM, Antwerp)

Results

PhD in Veterinary Sciences

Summary

Chapter 1 reviews the literature about the immunological aspects of taeniid cestode infections and the existing vaccines against *Taenia solium* cysticercosis in pigs. One of the most promising vaccines is TSOL18, a protein that has been identified in the oncosphere of *Taenia solium* and expressed as a recombinant molecule in *E. coli*. Repeated experimental trials have shown that this vaccine is able to protect up to 100% of the immunised pigs against a challenge infection with *T. solium*. Antibodies raised by the vaccine are capable of killing the parasite in *in vitro* cultures and it is believed that antibody and complement mediated killing of invading parasites is the major protective immune mechanism induced by vaccination with TSOL18.

The identification of the villages with a high risk of *T. solium* infection, which could subsequently be used in the vaccine trial, is reported in **chapter 2**. A survey was conducted in 150 households owning 1756 pigs in the rural areas of Mayo-Danay division in the far north region of Cameroon. A questionnaire survey was carried out to collect information on the pig farming system and to identify potential risk factors for *T. solium* cysticercosis infection in pigs. Blood samples were collected from 398 pigs with the aim of estimating the sero-prevalence of *Taenia solium* cysticercosis. The results showed that 90.7% of the pigs were free roaming during the dry season and that 42.7% of households keeping pigs in the rural areas had no latrine facility. Seventy six percent of the interviewed pig owners affirmed that the members of the household used open field defecation. ELISA for antigen and antibody detection showed an apparent prevalence of porcine cysticercosis of 24.6% and 32.2%, respectively. A Bayesian approach using the conditional dependence between the two diagnostic tests indicated that the true sero-prevalence of cysticercosis in Mayo-Danay was 26.6%. Binary logistic regression analysis indicated that the lack of knowledge of the taeniasis-cysticercosis complex and the absence of a pig pen in the household were associated with pig cysticercosis.

Chapter 3 reports the investigations that were undertaken to characterise whether the principal antibody specificities raised by TSOL18 in pigs were against linear or conformational determinants. TSOL18 was expressed in two truncated forms representing either the amino terminal portion or the carboxy terminal portion, with the two truncations overlapping in sequence by 25 amino acids. The original protein (designated TSOL18N⁺) and the two truncations (TSOL18N⁺-1 and TSOL18N⁺-2) were used in inhibition ELISA to determine their ability to inhibit the binding of protective pig antibodies to TSOL18. TSOL18N⁺ was shown to be capable of completely inhibiting the binding of pig anti-TSOL18N⁺ antibodies to TSOL18N⁺ in ELISA. However, neither TSOL18N⁺-1 nor TSOL18N⁺-2, either alone or combined, was capable of inhibiting any detectable amount of reactivity of pig

anti-TSOL18N[−] antibodies with TSOL18N[−]. It is concluded that the dominant antibody specificities, and likely the host-protective specificities, of TSOL18 are conformational epitopes.

Chapter 4 describes the development of an antibody detection test for the specific diagnosis of porcine cysticercosis. A fraction with a major band of 14 kDa was obtained from crude cyst fluid (CF) of *T. solium* cysticerci by 2-step chromatography. A first fraction isolated by gel filtration was purified using an anion exchange column on High Performance Liquid Chromatography (HPLC). Evaluation of the analytic sensitivity of this fraction (F3) was carried out in an antibody detection enzyme-linked immunosorbent assay (Ab-ELISA-F3) using serum samples from pigs experimentally infected with different doses of *T. solium* eggs. The cross-reactivity of F3 was evaluated with serum samples from pigs that were naturally or experimentally infected with *Taenia hydatigena*, *Taenia saginata asiatica*, *Fasciola hepatica*, *Trichinella spiralis*, *Metastrongylus apri*, *Trypanosoma congolense* or *Sarcoptes scabiei*, and with serum samples of rabbits hyper-immunised with cyst fluid of *T. hydatigena* or *T. solium*. Analysis of the specificity of the F3 showed that serum samples of pigs infected with other parasites did not recognise this antigen. Cross-reaction with *T. hydatigena* occurred in ELISA using CF as antigen, but the F3 antigen fraction was not recognized by the rabbit hyper-immune serum against *T. hydatigena*. Evaluation of the diagnostic sensitivity and specificity of the Ab-ELISA-F3 was done by a non-parametric Receiver Operating Characteristic (ROC) analysis using serum samples from Zambian and Cameroonian village pigs. The results from the ROC analysis yielded a low diagnostic value (area under ROC curve= 0.48) with the sera from the Zambian pigs while a relatively high diagnostic value was obtained with the sera from Cameroonian pigs (area under ROC curve= 0.78).

In **Chapter 5** the efficacy of the TSOL18 vaccine is assessed under field conditions in the Mayo-Danay district. Two hundred and forty 2-3 month old piglets belonging to 114 individual households were involved in the study. In each household one or more pairs of piglets were included, with one animal of each pair being vaccinated and the other acting as a non-vaccinated control. Vaccinated animals received two initial immunizations intramuscularly in the neck one month apart with 200µg TSOL18 plus 5mg Quil A. At the time of the second immunization both vaccinated and control animals received an oral dose of 30mg/kg oxfendazole. Vaccinated animals received a third immunization approximately 3 months after the first immunization. Antibody responses to the vaccine were assessed at different time intervals by ELISA. Necropsies were undertaken when the pigs were approximately 12 months of age. All parasites were counted in half of the body musculature and in the brain. Two hundred and twelve animals were available for necropsy at the end of the trial (110 vaccinated; 102 controls). Viable *T. solium* cysticerci were identified in 20 control pigs (prevalence 19.6%), including 14 animals that had estimated total body burdens of > 1000 cysticerci. No cysticerci were found in any of the vaccinated animals indicating that the vaccine provided a very high level of protection ($P < 0.0001$) against naturally acquired infection with *T. solium* in pigs. Combined application of TSOL18 vaccination and a single oxfendazole treatment in pigs is a simple and relatively sustainable procedure that has the potential to control *T. solium* transmission in endemic areas and, indirectly, reduce the number of new cases of neurocysticercosis in humans.

In **chapter 6**, the similarity of the antibody responses of pigs and mice to TSOL18 antigen is highlighted. Four IgG1 monoclonal antibodies (MoAb) were produced against the conformational epitopes of TSOL18. It was shown that pig antisera inhibit the binding of these MoAbs in a competition ELISA, indicating that pig and mouse antibodies against TSOL18 vaccine react with the same conformational epitopes. For this reason, monoclonal antibodies raised in mice immunized with TSOL18 could be a valuable source of antibodies for further characterisation of the host-protective epitopes of the vaccine. A monoclonal antibody-based inhibitive enzyme-linked immunosorbent assay (mi-ELISA) was developed. Serum samples of TSOL18-vaccinated and non-vaccinated pigs were used. In all the vaccinated and protected pigs screened at necropsy, anti-TSOL18 antibodies inhibited the binding of a monoclonal antibody (Mab25D12C1) specific to the conformational epitopes of TSOL18 antigen, suggesting an immune response that correlates with protection. This result was in agreement with the results obtained in an indirect ELISA, which showed that all the vaccinated and protected pigs had developed antibodies to the TSOL18 vaccine.

In **chapter 7** the efficacy of the TSOL18 vaccine is compared with that of other vaccines, which are currently being tested under field conditions. Recommendations are made for implementing a vaccination programme in Cameroon. Future research activities are suggested to improve our knowledge on the duration of the immunity of the vaccine and various other aspects of the vaccine production and delivery.