SHORT TERM SCIENTIFIC MISSION (STSM) – SCIENTIFIC REPORT

The STSM applicant submits this report for approval to the STSM coordinator
Action number: FA1408

STSM title: *Serological diagnosis of anisakid.*

STSM start and end up:15.10.2018 to 26.10.2018

Host: European Union Reference Laboratory for Parasites (EURLP),
Istituto Superiore di Sanità, Roma/ Italy

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1.PURPOSE OF THE STSM

Turkey is a peninsular country in which fishing habits and fishery product consumption are highly common. In the recent years, there is also an increase in the consumption of raw fish due to the increasing interest in the cuisine from Far Eastern countries.

Although there are publications showing the presence of anisakid parasites in fish and fishery products in the main European fishing grounds (1,2,3), there are no reports of human anisakidosis in Turkey. We think that this is caused probably by the lack of diagnostic tools and the low awareness of the infection.

The aim of the project is the development of diagnostic tools for the serological detection of anisakid parasites.

DESCRIPTION OF THE WORK CARRIED OUT DURING THE STSM

The first day (October 15th) I was introduced to the laboratory staff and then I visited the laboratories. The personnel showed me which protective personal devices I should use, how to use them, and what to do in case of an accident occurring. Furtherly, in collaboration with Dr. M. A. Gómez Morales and Dr. A. Ludovisi an agenda was defined on the basis of my expectancies, in which the main tasks were specified day per day as following:

15 of October: Third-stage larvae of *Anisakis* spp. (L3) were manually collected from the viscera of the cod fish (*Gadus morhua*) and extensively washed one by one in phosphate-buffered saline (PBS).Then L3 were washed in a 2 % acetic acid in PBS solution and then washed again in PBS. Further, L3 were transferred into Eppendorf® tubes (50 L3/tube) and stored at -20 °C.

16 of October: To prepare crude antigens (CA) and the allergen enriched fraction (AEF), 50 frozen L3 were suspended in 2 ml of PBS and then homogenized 5 times for 30 s and sonicated in ice 5 times for 60 s. The suspension was left overnight at 4 °C under magnetic stirring.

17 of October: The suspension was centrifuged at 1,500× g and at 4 °C for 15 min. The protein content of the centrifuged supernatant was measured by the Bradford method.

For the preparation of the AEF, the same procedure was carried out but after the last centrifugation, the supernatant was mixed with the same volume of ethanol (5 mL), incubated for 30 min at RT and centrifuged at 4,000 for 15 min. The supernatant was collected in a 15 ml Falcon tube and mixed with ethanol to increase the ethanol concentration to 66%. Then, the suspension was incubated for 30 min at RT and centrifuged at 4,000 for 15 min. The supernatant was discarded and the pellet was left at RT with open lid until the complete evaporation of the ethanol. Finally, the pellet was re-suspended in 250 µl PBS and stored at + 4 °C until determination of the protein concentration.

18 of October: The protein concentration was determined using the Bradford method. Standard curves were prepared with albumin bovine serum, and the optical density was determined at 595 nm by a microplate reader. The protein concentration was deduced by the GraphPad Prim 6®software.

CA resulted to have: 1.2 mg/ml

AEF resulted to have: 0.569 mg/ml

19 of October: Visual inspection of fish was performed to determine the presence of anisakid L3 in the market

visual inspection of fish to detect the presence of anisakid L3

Harvesting of L3 from fish muscle tissue

Homogenization of L3 with a mortar and pestle

Sheme 1: Summary of the 1st week with photos

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22 of October: Western blotting (Wb) methodology, listing devices, instruments, and chemicals needed were introduced. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as following:

- Proteins (20 µg of CA or 10 µg of AEF) were added to 4x loading buffer in such a way to reach a final volume of 30 µL/well, using analytic grade water. Proteins were electrophoretically separated by SDS-PAGE on 4-20 % pre-cast Novex Tris–Glycine Gels® (Invitrogen). Pre-stained MW standards (See Blue Plus2) were used (250, 148, 98, 64, 50, 36, 22, 16, 6 and 4 kDa) in each run (gel 1).

For Wb, proteins were passively transferred to nitrocellulose overnight at RT according to the s cheme of the Mini Trans-Blot (Bio-Rad).

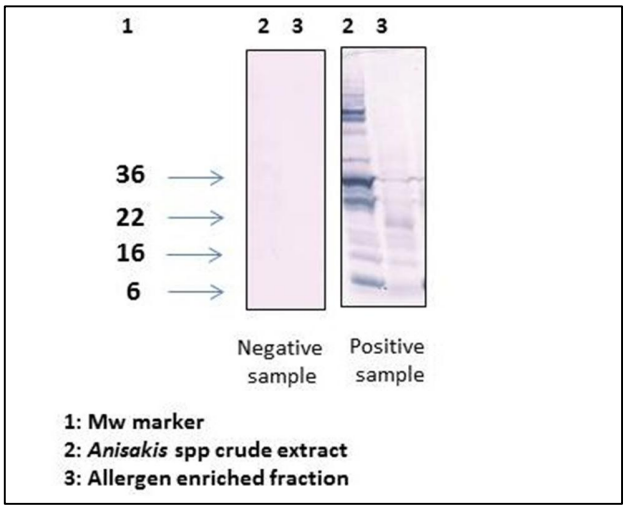
23 of October: At the end of the transfer, the nitrocellulose was removed and placed in a tray with Ponceau Red to verify the effective proteins transfer.

- After verification, nitrocellulose was washed with tap water to remove excess dye and cut in three strips using a scalpel.

- Then, strips were blocked in 3% Nonidet P-40 in Tris Borate Solution (TBS) for 1 hour in RT and after blocking they were furtherly washed 3 times for 5' at RT with TBS-Tween 20 solution (TBS-T).
- Serum samples were 1/5 diluted with diluting buffer (5% Fetal Calf Serum in TBS-T).
- Strips were placed into mini-incubation trays and incubated overnight at RT.
- New SDS-PAGE gel (gel number 2) was prepared and blotted overnight at RT.

24 of October: After incubation with the serum samples, strips from the gel 1 were washed 3 times for 5' at RT with TBS-T solution.

- 1/1000 diluted rat monoclonal antibody to human Ig-E was incubated with each strip for 1 hour in RT and washed 3 times for 5' at RT with TBS-T solution.
- 1/2500 diluted goat polyclonal antibody to rat IgG conjugated to alkaline phosphatase was incubated with each strip for 1 hour at RT and then washed 3 times for 5' at RT with TBS-T solution.
- BCIP-NBT (5-bromo-4-choro-3-indolyl phosphate-nitro blue tetrazolium) was the substrate used for revealing the proteins recognized by the sera tested. The reaction was stopped with tap water and results were interpreted. Discussion of the results.



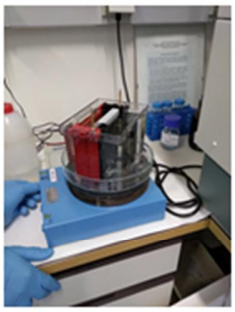
- Verification of protein transfer from the second gel was performed after overnight incubation. Then, strips were blocked and washed 3 times for 5' at RT with TBS-T solution. Then the strips were left to dry out and kept at RT in a plastic bag. These strips will be used to test Turkish human sera.
- New SDS PAGE gel (gel number 3) was prepared and proteins were transferred overnight at RT into nitrocellulose membrane.

25 of October: Verification of protein transfer from the third gel was performed after overnight incubation by diffusion. Then, strips were blocked and washed 3 times for 5' at RT with TBS-T solution. Then the strips were left to dry out and kept at RT in a plastic bag.

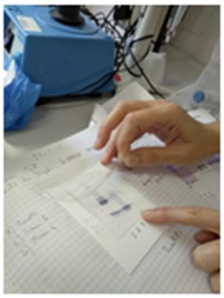
26 of October: General discussion regarding diagnostics for the serological detection of anisakid parasites.

All procedures were checked and wrapped- up and a report on STSM was carried out.

Prepared samples were loaded in the gel



Gel left to overnight incubation in RT by diffusion



Interpretation of results

Sheme 2: Summary of the 2st week with photos

In summary, my training was based on theoretical and practical approaches for the identification of *Anisakis* spp proteins recognized by specific IgE by Western Blotting. This STSM was very useful for my planned activities in the field of human parasitology

FUTURE COLLABORATIONS

Future collaborations between EURLP and Ege University are planned for the near future on the field of FBP, in particular on human diagnosis of parasitic diseases.

ACKNOWLEDGEMENTS

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References

1) Bušelić I, Botić A, Hrabar J, Staglič N, Cipriani P, Mattiucci S, Mladineo I. Geographic and host size variations as indicators of Anisakis pegreffii infection in European pilchard (Sardina pilchardus) from the Mediterranean Sea: Food safety implications. International Journal of Food Microbiology 266 (2018) 126–1322

2) Pekmezci GZ .Occurrence of Anisakis simplex sensu stricto in imported Atlantic mackerel (Scomber scombrus) represents a risk for Turkish consumers. Int J Food Microbiol. 2014 Aug 18;185:64-8. doi: 10.1016/j.ijfoodmicro.2014.05.018. Epub 2014 Jun 2

3) Zafer Pekmezci G , Yardimci B, Emek Onuk E, Umur S. Molecular characterization of Hysterothylacium fabri (Nematoda: Anisakidae) from Zeus faber (Pisces: Zeidae) caught off the Mediterranean coasts of Turkey based on nuclear ribosomal and mitochondrial DNA sequences. Parasitology International 63 (2014) 127–131.