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Human hantavirus infections in Hungary: occupational safety in risk group and clinical cases in the South-Transdanubian region between 2011-2015

PhD Thesis

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PÉCS, 2016

I. Scientific background, aims of the study

Roughly 60% percent of all human pathogenic agents (including bacteria, fungi, viruses and parasites) belongs to the category of zoonoses (Taylor et al., 2001); i.e. infectious diseases spreading from animals to humans. Several of the world's best-known diseases also belong to zoonotic agents, such as malaria, yellow fever, influenza, or Ebola itself, notorious for causing the its largest epidemic in 2015 in West Africa.

The emergence and spreading of zoonoses are regulated by several factors, of which growing human population is the most important one, implying other factors, like urbanization, land use and consequently deforestation. Some other factors can be associated with the rapidly advancing technology, i.e. global air travel, and in relation, the fashionable travel to "exotic areas," which are at the same time the usual home for the most dangerous zoonotic infections. Climate change is factor independent from human activities, but plays a decisive role in migration and in the distributional changes of host animal populations. All the above mentioned result in the increasing overlap of human and animal populations thus raising the chance for contracting zoonotic diseases.

The current work focuses on hantaviruses, marked representatives of zoonotic agents both worldwide and locally. Hantaviruses are spread by rodents; anyone can be infected regardless of gender or age. In Hungary, it is not a common disease regarding the annual scale (6-16 cases per year, Heyman et al. 2011), however, human cases might have a severe disease course. Hantavirus research in Hungary has a narrow range, we have few information on the clinical characteristics of the disease or about its local geographical emergence, especially among members of the "risk group." The latter incorporates persons (hunters, forestry workers), whose work activities connect them with hantavirus reservoir animals.

Among hantaviruses circulating in Hungary, Dobrava-Belgrade virus (DOBV) and Puumala virus (PUUV) are proven as pathogenic. Human infection diagnostics are based on immunologic assays globally. We also mainly utilized these kind of methods (ELISA, Western blot) during our study, supporting them with molecular methods (TaqMan reverse transcriptase PCR in our case) that enable the more precise identification of the infective agent.

The current work wants to provide an insight into the clinical characteristics of hantavirus infections in Hungary, as well as into the prevalence among risk group population. As such, our aims were the following:

- The producing of PUUV nucleocapsid protein (NP) in an *E. coli* expression system
- Optimization of an ELISA assay based on DOBV and PUUV NPs for large sample number screening
- Optimization of a Western blot (WB) assay to confirm ELISA results and for acute infection diagnostics
- Testing of acute-case samples with WB assay and TaqMan RT PCR
- ELISA screening of samples collected from risk group members between 2011 and 2013, confirming positives by WB
- Determining country-wide seroprevalence based on screening results
- The assessment of hantavirus prevalence among patients hospitalized with AKI in the region, in cooperation with the following clinics of the University of Pécs: 1st Department of Internal Medicine, Department of Infectology, and the 2nd Department of Internal Medicine and Nephrology Centre.

II. Materials and methods

Human samples

We present human sample collection in two parts. Sample collection from the risk group was carried out from volunteer hunters and forestry workers. Before blood sample collection, all volunteers filled out a questionnaire, which contained epidemiological data (Birth place and time, home); the time spent working "on terrain," earlier infections and related treatment, current medication and direct contact with animals. Sample collection was approved by the National Medical Scientific Ethical Board (ETT-TUKEB No#2213-0/2010-1018EKU).

Clinical samples were collected in cooperation with the University of Pécs, the 1st Department of Internal Medicine, Department of Infectology, and the 2nd Department of Internal Medicine and Nephrology Centre. Sera were collected prospectively from persons hospitalized with requirements for acute kidney injury (AKI):

- urinary abnormalities (few or absent urine)
- Increased leukocyte count
- serum creatinine concentration higher than 125 μmol/l upon hospital admission, which showed a 1.5-fold increase during a seven-day period

- Thrombocytopenia
- Increased serum transaminase levels
- The presence of protein and/or red blood cells in urine

Viral antigen expression

In ELISA and Western blot assays, DOBV and PUUV NP were used as viral antigens, since human immune response is the most prominent against these viral proteins. DOBV NP was readily available in frames of a previous work (Németh et al., 2011). PUUV NP was produced amplifying the NP gene coded by the S segment (1290 base pairs) from Sotkamo strain supernatant. The protein-producing construct (pET28a vector, 1290 bp insert) was transformed into E.coli BL21 Rosetta (DE3)pLysS cells, followed incubation at 37°C, 200 rpm in LB containing 30µg/ml kanamycin and 35µg/ml chloramphenicol as selective agents. Upon reaching log phase culture (OD_{600} = 0.8-1.0), cells were induced by 1mM IPTG overnight at 15°C. Protein purification was carried out under denaturing conditions using HIS Select® HF Nickel Affinity Gel column (Sigma Aldrich). Elution fractions were visualized by SDS-polyacrylamide gel electrophoresis.

ELISA and Western blot assays

96-well Maxisorp Nunc-Immuno Plates (Nunc) were used in ELSIA screening. Plates were coated with a combination of DOBV and PUUV antigens, followed by incubation for two hours at 37ºC. Excess antigens were removed by washing with phosphate-buffered saline (PBS) (pH 7.5), containing 0.05% Tween-20 (Sigma) (PBS-T). Microtiter plates were blocked for two hours at 37°C, using PBS containing 5% non-fat dry milk (Blotting-Grade Blocker, BIO-RAD) and 5% sucrose (Merck). Volunteers' sera were used in 1:100 dilution in PBS containing 2.5% non-fat dry milk. Serum samples of patients previously verified for DOBV and PUUV infections, respectively, were used as positive control. After incubation for one hour at 37°C, microtiter plates were washed with PBS-T. Horseradish peroxidase-conjugated rabbit anti-human IgG and IgM (DakoCytomation) were used as secondary antibodies, diluted to 1:4000 in PBS-T. Plates were incubated at 37°C for one hour, followed by washing again with PBS-T. Color development was allowed using 100 µl of 3,3',5,5'-tetreamethylbenzidine substrate (BD OptEIA), incubating at room temperature in the dark for 15 minutes. The reaction was stopped by the addition of 100 µl of 2M sulfuric acid, and optical density was measured on a microplate spectrophotometer (Thermo Electron Corporation) at 450 nm. The average optical density (OD) of negative control sera plus 3 times their standard deviation. We compared series by

calculating the ratio: observed OD/CO. A sample was considered positive if this ratio was at least 1.5. Ratios between 1.0 and 1.5 were considered ‘‘grey zone’’ values, while samples with ratio lower than 1 were considered to be negative.

All samples considered as positive (with high or “grey zone” OD values) were confirmed by immunoblot analysis, against both DOBV and PUUV nucleocapsid proteins. Nucleocapsid proteins of DOBV and PUUV were loaded into the wells of Mini-PROTEAN® Precast Gels (BIO-RAD). Polyacrylamide gel electrophoresis was carried out at 180V for 30 min. After electrophoresis, proteins were transferred to 0.45µm pore size nitrocellulose membranes (BIO-RAD), using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BIO-RAD), at 170 mA for 30 minutes. After blocking for 30 min with 5% non-fat dry milk (Blotting-Grade Blocker, BIO-RAD), sera were applied in 1:100 dilution using tris-buffered saline (TBS) (pH 7.5), containing 0.1% bovine serum albumin and 0.05% Tween-20 (Sigma). Horseradish peroxidase-conjugated rabbit anti-human IgG (1:2000 and rabbit anti-human IgM (1:1000 DakoCytomation) was used as secondary antibody. All incubation steps were executed at room temperature for 1 hour. Three consecutive 10 min washing steps were implemented using 1x TBS-Tween-20 (pH 7.5). Development was carried out using diamino-benzidine in 50 mM Tris (pH 7.5), containing 0.3% Nickel(II) chloride (Sigma).

TaqMan RT-PCR

RNA was extracted from 200µl human sera using, QIAamp Viral RNA Mini Kit (QIAGEN), according to the manufacturer’s recommendations. For detection of DOBV and PUUV RNA, published primers were used (Weidmann et al., 2005, Garin et al., 2001). PCR reaction was carried out using QIAGEN OneStep RT PCR Kit (QIAGEN).

Sequencing

Hantavirus-positive PCR amplicons were purified from 2.5% agarose gels using QIAquick Gel Extraction Kit (QIAGEN), kit according to kit protocol. Fragments were amplified for sequencing PCR with Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems), per manufacturer’s guidelines. The amplified, labelled DNA was precipitated with 3M sodium-acetate and absolute alcohol, followed by nucleic acid resuspension in Hi-Di^{TM} Formaide (Applied Biosystems). Samples were sequenced using the ABI Prism 310 Genetic Analyzer.

Statistical and phylogenetical analysis

Seroprevalence data for the distinct country regions were analysed by Chi square test. Differences in values were regarded significant at $p \leq 0.05$ value. Data were randomized using Microsoft Excel 2010 built-in algorithm.

III. Results

Viral antigens

Size of PUUV NP was determined as 55kDa and 59kDa for DOBV and PUUV viruses, respectively, using Agilent 2100 Bioanalyzer. ELISA and Western blot assays were verified comparing test results against the commercially available NovaLisa ELISA Kit (NovaTec), which resulted in above 90% specificity and sensitivity for both antigens in both ELISA and Western blot.

Clinical Samples

Between January 2011 and December 2015, 94 patient samples arrived in our laboratory from the cooperating clinics. Among these, hantavirus etiology was conformed in seven cases (7.4%), by simultaneously detecting hantavirus-specific IgM and IgG. Out of the seven acute cases, hantavirus RNA was only detected by TaqMan RT PCR in a single case, indicating a DOBV infection. Although immunological typing result may show cross-reactions, based on signal intensity, Western blot assay clearly indicated DOBV infections in the rest six cases. Leading clinical signs were fever, abdominal pain, vomiting and severely decreased urine output. A unified laboratory indicator parameter was increased serum creatinine concentration and thrombocytopenia. Additionally, for six patients, protein and red blood cells were also detected in urine.

Seroprevalence in the risk group

Between January 2011 and February 2013, totally 835 human sera were collected at 32 sites, belonging to 106 forestries. Among them, 750 were male and 85 female, with an average age of 45 years (range: 25-65 years). For data processing, we randomly selected 100-100 samples per region. Total seroprevalence was 5.5%, while regional values were the following:

2% in Danube-Tisza Plateau, 3% in South Transdanubian region, 5% in North Transdanubian region, and 10% in the North Hungarian Mountains.

IV. Discussion and closing thoughts

We summarize our results as follows. We were able to produce PUUV NP in adequate purity in an *E. coli* expression system. The produced protein is functioning during immunologic reactions in both denatured and renatured forms (we utilized the latter). The ELISA screening system was optimized using the mix of DOBV and PUUV proteins. An also optimized Western blot assay was used for confirmation of ELISA positives. Both immunological methods showed adequate specificity and sensitivity for testing the collected samples.

Data processing the clinical samples from persons with AKI provide an insight into hantavirus occurrence in the South-Transdanubian region of Hungary. Infections with prominent clinical signs and severe disease cases are rare, (7.4% of tested patients), at the same time, infections that require hospitalization are overwhelmingly caused by DOBV type. This observation is in accordance with other studies from the Balkans, where severe DOBV cases are reported usually, while PUUV infections often do not get diagnosed.

A total of 835 samples were collected from hantavirus risk group persons (hunters, forestry workers). We claim this sample amount to be fairly representative, considering similar studies and the size of Hungary. Regional seroprevalence was the highest in the North Hungarian Mountains (10%), which we put in association with the large populations of reservoir animals (mainly yellow-necked mouse) living in the area. Total seroprevalence was 5.5%, a value which we compared to a previous Hungarian study (Ferenczy et al., 2003). There, samples were collected randomly from person above 25
years of age from all areas of the country. Immunological testing (immunofluorescent assay) resulted in an overall seroprevalence of 10%. Comparing our results to the latter study some discrepancies arise. Still, we do not intend to falsify our hypothesis that hantavirus seroprevalence is higher in the risk group compared to background population. This hypothesis is verified by several studies (Antoiadis et al., 1987, Hukic et al., 2010, Heyman et al., 2011, Avšič Županc et al., 2014). A possible cause of similar maximum value from our study to the total seroprevalence of the 2003 study may be the for instance the different host animal

abundance in the two periods or the difference in sensitivity and/or specificity between the used immunological methods.

Finally, the points given as aims of the study we consider accomplished. We provide gap-filling hantavirus seroprevalence data regarding the Hungarian risk population, collected sample amount and results match to similar study results from the neighbouring countries, among which seroprevalence in Hungary can be considered as fitting in the medium range.

References

Antoniadis A, Le Duc JW, Daniel-Alexiou S (1987): Clinical and epidemiological aspects of hemorrhagic fever with renal syndrome (HFRS) in Greece. Eur J Epidemiol; 3(3):295-301.

Avšič Županc T, Korva M, Markotić A (2014). HFRS and hantaviruses in the Balkans/South-East Europe. Virus Res 2014 Jul 17;187:27-33.

Ferenczi E, Rácz G, Szekeres J, Balog K, Tóth E, Takács M, Csire M, Mezey I, Berencsi G, Faludi G (2003): New data for the public health importance of hantaviruses in Hungary. *Orv Hetil;* 144(109):467-74.

Garin D, Peyrefitte C, Crance JM, Le Faou A, Jouan A, Bouloy M. Highly sensitive Taqman PCR detection of Puumala hantavirus. *Microbes Infect* 2001;3:739–45.

Heyman P, Ceianu CS, Christova I, Tordo N, Beersma M et al. (2011): A five-year perspective on the situation of haemorrhagic fever with renal syndrome and status of the hantavirus reservoirs in Europe, 2005-2010. Euro Surveill; 16(36).

Hukic M, Nikolic J, Valjevac A, Seremet M, Tesic G, Markotic A (2010): A serosurvey reveals Bosnia and Herzegovina as a Europe's hotspot in hantavirus seroprevalence. Epidemiol Infect;138(8):1185-93.

Németh V, Madai M, Maráczi A, Bérczi B, Horváth G, Oldal M, Kisfali P, Bányai K, Jakab F (2011): Detection of Dobrava-Belgrade hantavirus using recombinant-nucleocapsid-based enzyme-linked immunosorbent assay and SYBR Green-based real-time reverse transcriptase-polymerase chain reaction. Arch Virol; 156(9):1655-60.

Taylor LH, Latham SM, Woolhouse ME (2001): Risk factors for human disease emergence. *Philos Trans R Soc Lond Series B Biol Sc;* 356(1411): 983–989.

Weidmann M, Schmidt P, Vackova M, Krivanec K, Munclinger P, Hufert FT (2005): Identification of genetic evidence for dobrava virus spillover in rodents by nested reverse transcription (RT)-PCR and TaqMan RT-PCR. *J Clin Microbiol;* 43(2):808-12.