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Original article

The survey of wild birds for West Nile virus in Poland

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Abstract

Two thousand one hundred and forty birds belonging to 39 different species from different locations in Poland were examined. The study has taken place from the early spring till late autumn 2007-2010 when the activity of the mosquitoes was the highest. The brain samples were taken from the birds and whole cellular RNA was isolated, then the RT-PCR and NRT-PCR were performed to detect the presence of West Nile virus (WNV). The obtained results were confirmed by the commercial WNV Kit. No genetic material of WNV was found in the examined samples.

Key words: West Nile virus, wild birds, RT-PCR, NRT-PCR

Introduction

During the last years acute infections appear more and more frequently in humans. The infections are characterised by high pyrexia and morbidity caused by the factors so far acknowledged as pathogenic only for the animals. One of the factors is West Nile virus which can cause zoonosis-West Nile fever (WNF). West Nile virus is on the list of the World Organisation for Animal Health (OIE) as the neurotropic factor causing the disease under the obligation to notify.

WNV is an arbovirus, which belongs to the *Flaviviridae* family, genus *Flavivirus*. The ssRNA+ genome of the virus contains a single open reading frame from 11,000 to 12,000 nucleotides. The genome of the virus consists of seven genes of non-structural proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5, and three genes of structural proteins: glycoprotein E, core protein C, and pre-membrane protein prM (Brett et al. 2007).

The tropical and migrating water birds that belong to different species are the main reservoir of the virus (Hubalek et al. 2008). In the world, about 135 different bird species were infected by the virus. The blood-eating insects are the virus vector. Usually the virus circulates between wild birds and mosquitoes in closed cycle and can be carried by the birds during their migrations. There is the possibility of the virus transfer from the infected migration birds to wintering wild birds in Poland.

The virus occurs in many countries around the World, also in 20 countries of the European Union, including close neighbors of Poland, where the presence of the virus has been confirmed (Platonov et al. 2001, Fyodorova et al. 2006, Linke et al. 2007, Hubalek et al. 2008a,b). The presence of WNV antibodies was also confirmed in serum samples from wild birds and humans in Poland (Hubalek et al. 2008, Wegner and Hubalek 2008). In 2010 human morbidity and mortality caused by WNV infection were

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reported in Greece, Russia, Romania, Italy, and Israel (http://ecdc.europa.eu 2011).

The aim of the study was the investigation of the WNV presence in wild birds in Poland using own developed and optimised RT-PCR and NRT-PCR methods.

Materials and Methods

Birds. 2140 wild birds brains from 39 species were examined. The birds were delivered to the laboratory from various locations in Poland, 2007-2010 (Fig. 1). The examined birds comprised: 696 wild ducks (*Anas platyrhynchos*), 33 common buzzards (*Buteo buteo),* 629 garganeys (*Anas querquedula*), 48 hooded crows (*Corvus corone cornix)*, 38 eurasian coots (*Fulica atra*), five common ravens (*Corvus corax)*, 115 common pheasants (*Phasianus colchicus*), three western capercaillies (*Tetrao urogallus)*, four willow warblers (*Phylloscopus trochilus)*, 16 common swifts *(Apus apus),* 247 grey partridges (*Perdix perdix*), three Eerasian bitterns (*Botaurus stellaris)*, 39 european robins (*Erithacus rubecula*), five common kingfishers (*Alcedo atthis)*, 14 common chiffchaffs, (*Phylloscopus collybita*), 14 whitethroats (*Sylvia communis)*, three spotted flycatchers (*Muscicapa striata*), two hawfinches (*Coccothraustes coccothraustes),* eight pied flycatchers, (*Ficedula hypoleuca)*, six montagu's harriers *(Circus pygargus),* one dunnock *(Prunella modularis immaturus)*, five common Kestrels (*Falco tinnunculus)*, one european magpie (*Pica pica),* eight common cuckooes (*Cuculus canorus*), seven thrush nightingales (*Luscinia luscinia)*, one common kestrel (*Falco tinnunculus*), 12 wood warblers (*Phylloscopus sibilatrix)*, three jackdaws (*Corvus monedula)*, 108 great tits *(Parus major*), four hooded crows (*Corvus cornix*), two chaffinches (*Fringilla coelebs*), three song thrushes (*Turdus philomelos*), two winters wren (*Troglodytes troglodytes*), two goldcrests, (*Regulus regulus*), one skua (*Stercorarius pomarinus)*, three white storks (*Ciconia-ciconia*), 17 european herring gulls (*Larus argentatus)*, one whitethroat (*Sylvia communis),* and 45 goshawks (*Accipiter gentili)*.

Material. Brain samples collected from the birds were homogenised with addition of RNase-free water (Qiagen, Germany) and centrifuged in 2.0 ml vials at 15,000 g for 5 minutes. Total RNA was isolated with RNA Mini Kit (Qiagen, Germany) according to manufacturers' protocol. Isolated RNA was stored at -80°C with addition of RNase inhibitor (Promega, USA) for further research.

Controls. The following positive controls were used: K1 – conservative fragment 3'NCR (Non-Coding Region) of virus genome from ATCC WNV NY-99 strain from commercial WNV kit (Prodesse, USA) and K2 – RNA from seagull brain artificially infected with IS-98-ST1 strain, WNV lineage 1, acquired from reference laboratory AFSSA, France. Negative controls were whole cell RNA isolated from chicken embryo fibroblasts (CEF – SPF) and deionised water.

In order to characterise RT-PCR specificity, genetic material of tick-borne encephalitis virus, Usutu virus, reovirus S1133 strain, and duck hepatitis virus were used.

Primers. Oligonucleotide sequences used in RT-PCR were prepared on the basis of conservative sequence of 3'NCR of ATCC WNV NY99 strain (GeneBank accession number: DQ211652): WNV3 – 5'-GCC ACC GGA AGT TGA GTA GA-3' and WNV4 – 5'-CTG GTT GTG CAG AGC AGA AG-3'. Such primers allowed amplification of 450 bp fragment complementary to 3'NCR of WNV genome. Primers used in NRT-PCR were prepared on the basis of predicted sequence of amplicon resulting from RT-PCR. NRT-PCR primers were: WNV5 – 5' AAA GCC CAA TGT CAG ACC AC 3' and WNV6 – 5' TAG TCC TTT CGC CCT GGT TA 3'. NRT-PCR product was 150 bp long.

RT-PCR. RT-PCR was performed in a T Professional Basic Gradient thermocycler (Biometra, Germany). Reverse transcription and amplification reaction was performed with use of One Step RT-PCR kit (Qiagen, Germany). RT-PCR mastermix contained 5 µl of 10x buffer, 2 µl of dNTP (10 mM), 1.5 µl of each primer (10 mM), 2 µl of enzyme mix, 5 µl of Q-solution, and deionised water up to total volume of 45 µl. Five microlitre sample of RNA was added to the mastermix. Reaction conditions were as follows: reverse transcription at 50°C for 30 minutes, initial denaturation at 95°C for 15 minutes, 35 cycles of: denaturation at 95°C – 45 s, annealing at 54°C – 45 s, elongation at 72°C – 1 min. Final elongation was at 72°C for 10 minutes**.**

Nested RT-PCR. The reaction was performed in total volume of 25 µl. Master mix contained 2.5 µl of 10x buffer, 1 µl of dNTP (10 mM), 0.75 µl of each primer (10 mM), 1 µl of enzyme mix, 2.5 µl of Q-solution and deionised water to total volume of 22.5 µl. 25 µl of RNA sample were added to mastermix. Reaction conditions were as follows: initial denaturation at 95°C for 15 minutes, 35 cycles of: denaturation at 95°C – 45 s, annealing at 59°C – 45 s, elongation at 72°C – 1 min. Final elongation was at 72°C for 10 minutes.

Electrophoresis. Product of NRT-PCR was elektrophoresed in 2% agarose gel at 120V for 50 minutes. Afterwards, the gel was stained with ethidium bromide (1 µg/mL) and visualised under UV (302 nm). Product sizes were estimated by comparison with

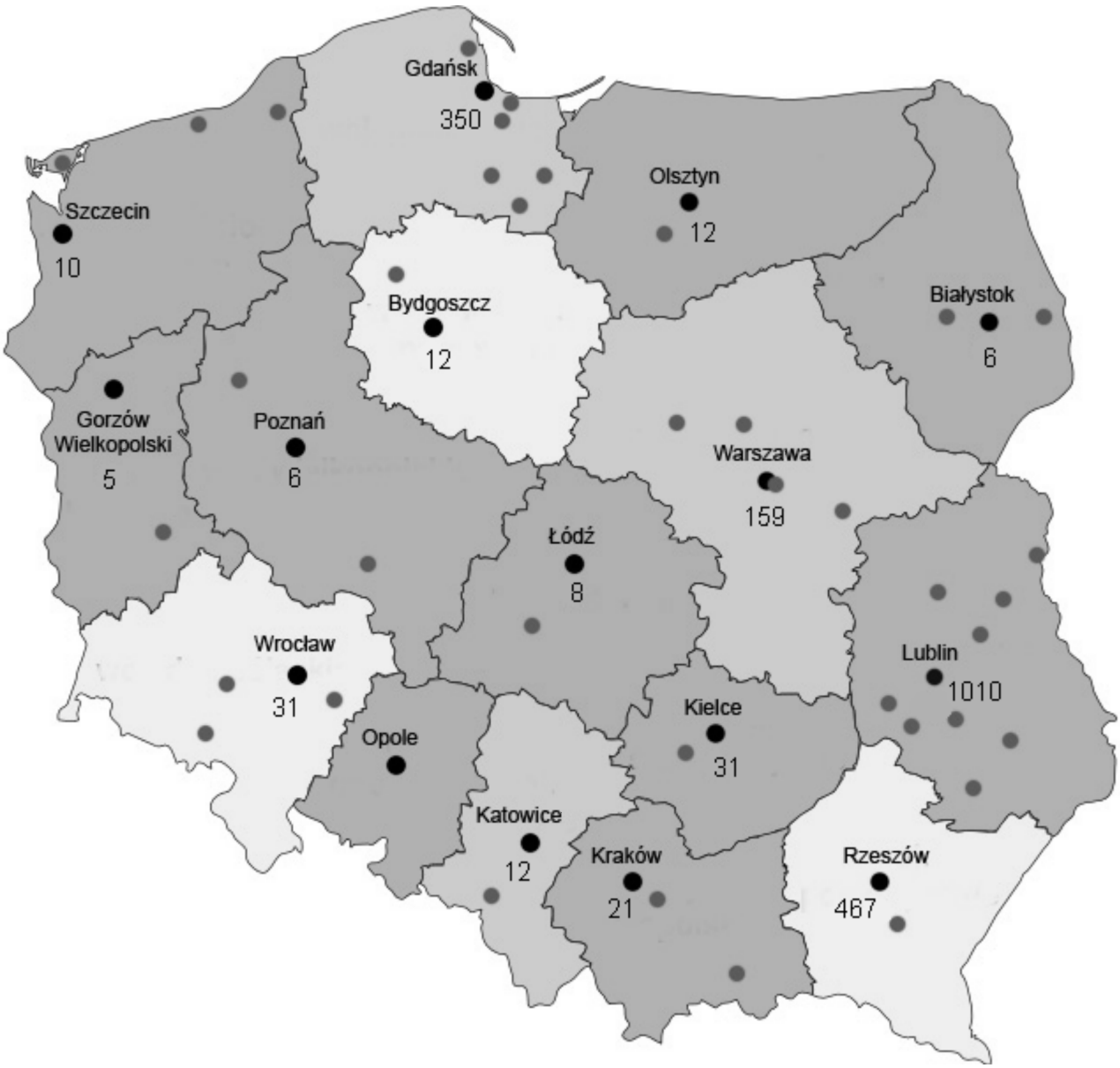

Fig. 1. Places of the collection of samples from wild birds in Poland.
● – grey points – the sites of wild birds catching

mass ruler (Fermentas, Germany) and photographed. In order to confirm the results the samples were also examined with WNV kit (Prodesse, USA) according to manufacturers' protocol.

Results

In several cases only heads of the wild birds were brought to laboratory. Therefore, no pathological examinations were performed. However, the hunters who delivered the samples, informed that all birds were in good general conditions and there were no pathological changes in their internal organs. On the other hand, the ornithological stations and wild birds rehabilitation centers noted several causes of morbidity and mortality of the examined birds: fractures of legs or wings, starvation, poisoning and other reasons.

The examination of birds; brains did not show any pathological changes. No amplicon of 450 bp (RT-PCR) nor 150 bp (NRT-PCR) characteristic for RNA of WNV was found in the brain tissue. Positive results were obtained only in case of positive controls. The results of RT-PCR and NRT-PCR were identical with the results obtained with commercial WNV kit.

Optimised RT-PCR and NRT-PCR methods were highly specific and did not give any positive reactions with close related flaviviruses such as tick-borne en-

cephalitis virus, Usutu virus, and other common viruses infecting poultry like reovirus strain S1133 and duck virus hepatitis.

Discussion

During last years, it is noticeable that WNV spreads in the countries with moderate climate. The most common vector of the virus are mosquitoes from the *Culicidae* family (Juricova et al. 1998, Epstein 2001, Glavitis et al. 2005, Hubalek 2008), and nowadays six of the mosquito species exist in Polish climate zone (Hermanowska-Szpakowicz et al. 2006, Hubalek et al. 2008a). In the nineties, Juricova (1998) using haemaglutination inhibition assay demonstrated WNV antibodies in 12.1% of house sparrows (*Passer domesticus*) and 2.8% of Eurasian tree sparrow (*Passer montanus*), which live in Kampinos forest area in Poland. In subsequent years, WNV antibodies were found in three storks (*Ciconia ciconia*), one crow (*Corvus corone cornix*), and one mute swan *(Cygnys olor)* in other part of Poland.

WNV antibodies were found in elderly febrile woman (Hermanowska-Szpakowicz et al. 2006) and in healthy forest workers from Swietokrzyskie (28.85%) and Podlaskie (34.14%) voivodeships (Kondrusik et al. 2007). The examination of twenty-four serum samples from hospitalised patients with the symptoms of tick-borne encephalitis showed WNV antibodies in 14 samples (own research, not published yet).

In our study, there was no possibility to perform the serological examination of wild birds, and because of that reason the study was based on the RT-PCR and NRT-PCR for the detection and identification of WNV in the tissue culture and cerebrospinal fluid samples from humans and animals (Brise et al. 2000, Lanciotti et al. 2000, Shi et al. 2001, Kauffman et al. 2003, Bhatnagar et al. 2007, Niczyporuk and Samorek-Salamonowicz 2009). The specific oligonucleotide primers were used to target conservative 3'NCR fragment of the WNV genome. This region exists in all WNV strains without many mutations. The sensitivity of our method was 10^{-5} ng/μL of DNA detected (Niczyporuk and Samorek-Salamonowicz 2009). Our results were compared with the results obtained with commercial WNV Kit with the sensitivity of 1.5 PFU/1 mL for the validation process. The specificity of our method was confirmed by the use of a genetic material from close-related flaviviruses and other viruses commonly found in poultry. Despite correct use of RT-PCR and NRT-PCR methods, no genetic material of WNV in the examined wild birds in Poland was detected. The results were not influenced by the factors like: birds species, their origin, age or health birds (shot or naturally dead). However, on the basis of epidemiological situation in Europe, the role of wild birds in virus transmission, and results of previous serological examinations of humans and birds (Juricova et al. 1998, Hermanowska-Szpakowicz et al. 2006, Kondrusik et al. 2007, Hubalek et al. 2008, Wegner and Hubalek 2008) we can suspect there is the high possibility that the virus is already present in our ecosystem.

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