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PhD SCHOOL OF ANIMAL AND AGRICULTURAL ENVIRONMENT SCIENCES

SUMMARY OF PhD THESIS

Examination of avian botulism on the area of Lake 'Kis-Balaton'

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1. Preliminaries and aims of the research work

In the light of the number of affected species and mortality data, avian botulism caused by the toxigenic obligate anaerobe bacterium *Clostridium botulinum*, is the most significant disease of waterbirds worldwide. Between 1994 and 1997, more than four million waterfowl died in the USA and Canada only. Even one Californian outbreak in 1996 was enough to kill nearly 15% of the western population of American White Pelicans (*Pelecanus erythrorhynchos*) /ROCKE, 2006/.

Beside the economic loss, intangible loss derived from the death of protected and endangered species can also be huge. In this respect, nature reserves where avian botulism periodically recurs has great importance as well as the region of Lake 'Kis-Balaton' in Hungary, a part of Balaton National Park where avian botulism killed thousands or even tens of thousands of waterbirds in a single outbreak /MAGYARI; personal communication/.

It has been known for a long time that various biotic and abiotic factors could favourably influence the occurrence of the epizootics. As WOBESER /1987/ claimed: 'no single set of environmental conditions that can be associated with all outbreaks of botulism', but he named five key factors: presence of spores, favourable environmental conditions for bacterial growing, infection of *Clostridium botulinum* by specific bacteriophages, ingestion of the toxin by susceptible birds and spreading of bacteria and toxin by appropriate vectors.

In the light of these findings, diseases occurring under (seemingly) unfavourable environmental characteristics – and vice versa – can be quite puzzling.

Relying upon Hungarian and international experiences it is statable that we are facing a complex, changing system, habitat by habitat in which we must also take account the effect of other, yet unknown or disregarded factors beside the key elements.

Although the etiology of the disease is known for almost a century, difficulties in isolation of *Clostridium botulinum* from natural samples make avian botulism mysterious from the side of the pathogen, too. For instance, selective medium is not available to date to separate proteolytic type **C** *Clostridium botulinum* strains from the accompanying flora. Furthermore, many methodological complications derived from the obligate anaerobic characteristics of these bacteria.

Evading this difficulty, toxin detection is used instead of demonstration of bacteria in routine diagnostic work. Up to the present, toxin neutralisation test based on mouse inoculation is the only, widely accepted and applied method either in human or animal botulism cases. However, the misgivings about animal tests urge new, more suitable techniques whether ethical or financial or methodological respects from time to time. Beside immunological-

based assays, polymerase chain reaction (PCR)-based methods – which increasingly gaining ground in confirmation of avian botulism outbreaks – could be like these. At the same time, it should not forget that techniques mentioned above are reliable only when they are combined with each other.

The most important aim of this research program is to reveal the connection between various biotic and abiotic environmental factors and avian botulism outbreaks on the area of Lake 'Kis-Balaton' where the disease is thought to be endemic. The examinations involve a comparison between water temperature, organic matter content (COD – chemical oxygen demand), pH, conductivity and water-soluble oxygen (WSO) levels arising from high and low botulism risk sampling points and periods as well as air temperature and distribution of precipitation of the area.

Furthermore, we aimed to detect and isolate the etiological agent *Clostridium botulinum* from mud samples derived from the above mentioned wetland site by classical microbiological and various molecular techniques including conventional and real-time PCR reactions. Analysis of applicability of these molecular methods in diagnosis of the disease has been also performed.

Because of the poor Hungarian literature of this topic, we tried to discuss avian botulism in its entirety. The results of this research project may lead to a better understanding of the development of avian botulism, the more precise prediction of outbreaks as well as the reduction of economical and intangible loss caused by them.

2. Materials and methods

Statistical analysis of environmental factors

First of all, the whole territory was divided into high and low botulism risk (HR and LR) areas, according to the sites of former outbreaks. Five-five sampling points, located in HR and LR areas were chosen, respectively.

(Bi)weekly collected water-quality parameters: water temperature, pH, conductivity, water-soluble oxygen (WSO) and organic matter content (chemical oxygen demand (COD)) of these points were examined. According to the annually botulism mortality reports, five-five HR (avian losses associated with botulism) and LR years (without botulism-like mortality) were selected. Since the mortality peak has been usually developed between July and September in this region, these periods were observed in each year.

Four groups were formed by combination of the sampling points and years. One-way analysis of variance (ANOVA) and paired-samples t-test to every water-quality parameter of the groups was performed by SPSS 16.0 statistical software. By processing the mean values of

each parameter, discriminant analyses were also used to determine the statistical separation between the groups.
Meteorological data of the examined periods including daily minimum, maximum and mean temperatures as well as daily rainfall values were also evaluated by one-way ANOVAs and paired-samples t-tests.

*Detection of type **C** Clostridium botulinum organisms in mud samples* derived from *Lake 'Kis-balaton' by PCR reactions*

Nested PCR reactions according to WILLIAMSON et al. /1999/

Sediment samples were collected from five areas located in Lake 'Kis-Balaton', that have persistently shown high risk potentials for avian botulism outbreaks. Approximately 100 g from the top 10 cm of bottom sediment was collected and then frozen at -20°C within 4-5 h of collection. Total DNA was isolated from 0.25 g of sediment, using PowerSoil™ DNA Isolation Kit (MoBio, USA), according to the manufacturer's recommendations.

PCR assays (20 µl reaction mixtures) were performed with a DNA thermal cycler (RoboCycler; Stratagene, USA). The amplification reaction mixtures contained 2 µl of 10x PCR buffer (1x is 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 8.8] and 0.1% Triton X-100; Finnzymes, Finland), 2.0 mM of each desoxynucleoside triphosphate, 20 pM of each primer (Metabion, Germany), 0.4 U of DynaZyme DNA polymerase (Finnzymes, Finland) and 20 ng of DNA template in a 0.2 ml thin-walled polypropylene PCR tube (AHN, Germany) without a layer of oil. In nested PCR reactions, 2 µl of the initial PCR product was used. Purified bacterial DNA derived from toxigenic strains 468 Type **C** and 2145 Type **C** were used as positive controls.

The resulted PCR products were separated by 1.5% agarose gel electrophoresis (HU25; Scie-Plas, UK) in 0.5x TBE buffer. The amplification products were stained for 20 minutes in ethidium bromide solution then visualised and photographed with a UV transilluminator (Gene Genius Bio Imaging System; Syngene, Cambridge, UK).

At the time of the collection of sediment samples, two mallards (*Anas platyrhynchos*) were died on the area of Lake 'Kis-Balaton' just after they recovered from avian botulism. The content of their caeca was also sampled and investigated by the method described above.

In addition, a small amount from all of the native sediment and caecum samples were cultured in Schaedler broth (Scharlau, Spain) for 4 days at 30°C under anaerobic conditions. Before cultivation, one set of the samples was placed into 70°C waterbath for 15 minutes to eliminate vegetative cells. Heat-treated and untreated samples were then streaked on the surface of Schaedler agar plates (Scharlau, Spain) and were stored in anaerobic jars for 3 days at 30°C. Colonies isolated from agar plates were analyzed with the same PCR method as the uncultivated samples.

Conventional PCR reactions according to FRANCIOSA et al. /1996/

DNA isolates used in these examinations came from the same, cultivated and uncultivated sediment and caecum samples as above. Composition of 50 µl PCR reaction mixtures was similar to FRANCIOSA et al. /1996/ with one exception: our PCR buffer contained 1% Triton X-100 beside $MgCl_2$.
Ingredients of the PCR assays were: 10x PCR buffer (1x is 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 8.8] and 0.1% Triton X-100; Finnzymes, Finland), 200 µM of each desoxynucleoside triphosphate, 1 µM of each primer (Metabion, Germany), 0.4 U of DynaZyme DNA polymerase (Finnzymes, Finland) and 2 µl of DNA template in a 0.2 ml thin-walled polypropylene PCR tube (AHN, Germany). Purified bacterial DNA derived from toxigenic strains 468 Type **C** and 2145 Type **C** were used as positive controls.

The resulted PCR products were also separated by 1.5% agarose gel electrophoresis (HU25; Scie-Plas, UK) in 0.5x TBE buffer. The amplification products were stained for 20 minutes in ethidium bromide solution then visualised and photographed with a UV transilluminator (Gene Genius Bio Imaging System; Syngene, Cambridge, UK).

Modified real-time PCR reactions according to FRANCIOSA et al. /1996/

The positive experiences with the above mentioned conventional PCR method were led to its upgrading to a real-time technique. In these examinations, the primer pair designated by

FRANCIOSA et al. /1996/ was used in a LightCycler- and SYBR Green I-based real-time environment including melting analysis. The 20 µl volume amplification reaction mixtures contained 2 µl of PCR buffer (including enzyme and desoxynucleoside triphosphate), 2.4 µl of $MgCl_2$, 2 µl of each primers (in 20 pM final concentration), 9.6 µl of PCR water and 2 µl of DNA template, using LightCycler®FastStart DNA Master SYBR Green I kit (Roche, Germany). The resulted PCR products were separated by chip electrophoresis using Agilent 2100 Bioanalyzer system (Agilent, USA).

Purified bacterial DNA derived from toxigenic strains 2145 Type **C** and 2279 Type **C** were applied as positive controls.

LUX primer-based real-time reactions

C. botulinum BoNT/C_1 gene specific LUX primers were designated by Invitrogen D-LUX primer designing tool (http://escience.invitrogen.com/lux/index.jsp). Checking of the results by Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Oligo Analyzer (http://www.softpedia.com/get/Science-CAD/Oligo-Analyzer.shtml) programs, increasing of GC ratio and melting points of the primers would need. For the sake of this goal, slightly modified primers were designed and manufactured (Kromat, Hungary) with the help of the programs mentioned above. The reverse, fluorogenic primer was synthesised on the model of LUX primers labelled with FAM (phosphoramidite) fluorophore while forward primer was unlabeled.

20 µl reaction mixtures and real-time protocols on LightCycler® 1.5 instrument were refined constantly. PCR mixtures consisted of PicoMaxx High Fidelity PCR System – included the enzyme in concentration of 2.5 U/µl and 10x reaction buffer (Agilent, USA), $MgCl_2$ (Thermo Scientific, USA), dNTP (Fermentas, USA), BSA (10%; Calbiochem, USA), forward and reverse primers as well as PCR water (AccuGENE; Lonza, Switzerland). In these experiments, 2145 and 2279 *Clostridium botulinum* Type **C** strains were used as positive controls.

*Isolation of type **C** Clostridium botulinum organisms from mud samples derived from Lake 'Kis-balaton' by culturing*

The aim of this work was the isolation of toxigenic type **C** *Clostridium botulinum* strains from sediment samples coming from various points of Lake 'Kis-balaton' using classical microbiological methods.

From the mud samples, 3.5 g (in wet weight) were placed into 10-10 tubes containing DRCM or Holmann bouillon. After that, the tubes were closed with molten paraffin, placed into 70°C waterbath for 15 minutes to eliminate vegetative cells and incubated for 24-48 hours at 37°C. 50 µls of the samples were then streaked on the surface of modified McClung-Toabe EYA supplemented with $FeCl_3$ and CCFA agar plates and were stored in anaerobic jars for 2 days at 37°C using aerobe controls.

Colonies showing lecithinase and lipase activity were incubated in TPGY broth at 37°C for 48 hours. After that, morphological examinations of the colonies and microbes were performed using colony- and phase-contrast microscope.

To investigate hemolysis, 50 µl from the cultures growing in TPGY broth were streaked onto Columbia blood agar plates containing 3% of sheep blood and cultivated under anaerobic circumstances at 37°C for 48 hours using aerobe controls. Colonies showing β-hemolysis were then cultured at 37°C for 24 hours in TPGY and Holmann broths and investigated by API 20A system (bioMérieux, France) and conventional PCR reactions according to FRANCIOSA et al. /1996/.

3. Thesis statement

a. In some cases, significant ($P<0.05$) differences were found between several environmental factors (air- and water temperature, pH, water-soluble oxygen and organic matter content) and the occurrence of avian botulism outbreaks.

The values of these parameters were recorded periodically in high (HR) and low (LR) avian botulism risk areas of Lake 'Kis-Balaton'. Data derived from both HR and LR years (with or without documented botulism-caused avian losses) were processed.

b. False positive results were detected in the course of nested PCR work according to WILLIAMSON et al. /1999/. 16S rDNA analysis of the separated PCR products verified the presence of *Escherichia coli* organisms.

c. The conventional PCR method described by FRANCIOSA et al. /1996/ was upgraded to a LightCycler-based real-time technique including melting analysis to get a faster, more simple and sensitive reaction.

d. In the light of the results, applicability of these molecular techniques in diagnosis of avian botulism cases was also investigated.

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