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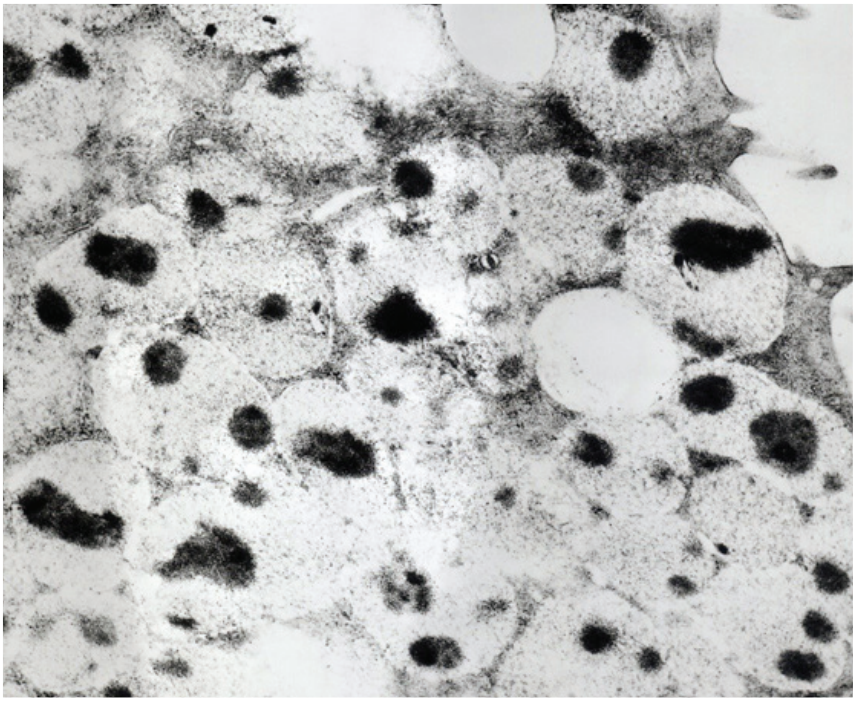

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Review Article

INTEGRATED USE OF DIFFERENT FISH RELATED PARAMETERS TO ASSESS THE STATUS OF WATER BODIES

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Summary: Freshwater bodies receive high levels of different toxicants. Since fish are typically situated at the top of aquatic food chains, they have a good potential to be used as indicators of water pollution. Assessment of the presence of pollutants and their toxicity can be efficiently performed by the combined use of analytical chemistry, bioassays and applied mathematics. In this study, we present the general approach of the integrated use of different fish related parameters to assess the status and pollution levels of waterbodies. We discuss our previous experiences within the field of analytical toxicology, toxicological pathology and toxicity testing, as well as biomathematical and statistical methods that are able to provide for integration of results acquired by each of the specific methods. We discuss advantages and shortcomings of each of the methods, and present necessary future steps in the method development. Since the industrial and domestic wastewaters in Serbia are still not processed before being released into watercourses, they pose a serious risk for aquatic ecosystems and public health. Consequently, described pollution indicators and genotoxicity parameters represent an essential tool for efficient monitoring of aquatic ecosystems. Methodological approach presented here might be of interest for scientists and managers dealing with the ecotoxicological research and monitoring of freshwater ecosystems.

Key words: freshwater fish; heavy metal; histopathology; genotoxicity; PCA; CDA

Introduction

Freshwater ecosystems are nowadays exposed to high anthropogenic impacts from untreated or poorly treated industrial and communal wastewaters, runoff from agricultural lands and mining sites, as well as from numerous other sources connected with human activities (1, 2). As a result, freshwater bodies receive high levels of different toxicants. Assessment of the presence of pollutants and their toxicity can be efficiently performed by the combined use of analytical chemistry, bioassays and applied mathematics. In such an approach, analytical toxicology would provide information regarding the identification and assay of toxicants in environmental and biological materials, toxicological pathology would assess the effects of toxicants and their metabolites on cell and tissue morphology, while genotoxicity testing would use living systems to estimate genotoxic effects. Since these

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approaches commonly deal with a substantial amount of acquired data, they also require the use of advanced biomathematical and statistical methods for data analysis.

Fish have a potential to be utilized as indicators of water bodies' pollution status, as different fish species occupy different habitats and belong to different trophic levels (3, 4). According to the Water Framework Directive (EU Directive 200/60/C), fish are one of the most important biological quality elements for the assessment of the ecological status of water bodies. However, only a few fish related parameters, such as fish species composition, abundance and age structure, have been commonly included in such assessments so far.

In this study, we present the general approach of the integrated use of different fish related parameters to assess the status and pollution levels of waterbodies. We present our previous experiences within the field of analytical toxicology, toxicological pathology and toxicity testing, as well as biomathematical and statistical methods that are able to provide integration of the results acquired by each of the specific methods. We discuss advantages and shortcomings of each of the methods, and present necessary future steps in the method development.

Analytical toxicology

Heavy metals are considered to be among major pollutants in the Danube River basin in Serbia (5). Since fish are typically situated at the top of aquatic food chains, they are able to accumulate large amounts of some metals (6). Heavy metals can be either ingested through food or absorbed from the water through gills and skin (7). Following their absorption, heavy metals and trace elements demonstrate tissue specific accumulation patterns in fish (8, 9).

The two most widely used techniques for heavy metal and trace element analyses are Atomic Absorption Spectrometry (AAS) and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Each of the two methods is characterized by certain advantages and drawbacks when compared with the other one. Although ICP-OES has better detection limits than the flame-AAS, it has significantly lower sensitivity than the graphite furnace-AAS. Both methods are generally considered as inexpensive, although ICP-OES has somewhat higher running costs of analysis. On the other hand, ICP-OES provides a much greater speed of the analysis, when compared with the standard AAS methods. This feature represents an important advantage in the field of analytical toxicology and environmental monitoring, which typically deal with studies that are based on analyzing a large number of samples. As a result, ICP-OES is gaining ground in ecotoxicological research as a more dominant method, and will be further described here.

Sample preparation procedure for the ICP-OES analysis is as follows: caught specimens are dissected and samples of different tissues are quickly removed, washed with distilled water and stored on -20°C prior to analysis. Samples are freeze-dried, and 0.2-0.5 g dry weight sample portions are subsequently processed in a microwave digester, using 6 ml of 65% HNO_3 and 4 ml of 30% H_2O_2 at a 100–170°C temperature program. The problem with the potential presence of trace elements in chemicals used for digestion is commonly resolved by using a number of blank samples, while the analytical process quality can be controlled by the use of reference material. Following the cooling to room temperature, digested samples are diluted with distilled water to a total volume of 25 ml.

Our previous research within this field has been mainly focused on the assessment of heavy metal and trace element accumulation levels in different localities (3), in different fish species (4, 8, 9), along the aquatic food chains (i.e., in fish and piscivorous birds; 7), as well on general accumulation patterns in different fish tissues. Research included assessment of the following heavy metals and trace elements: Ag, Al, As, B, Ba, Cd, Co, Cr, Cu, Fe, Hg, Li, Mg, Mn, Mo, Ni, Pb, Si, Se, Sr and Zn. The major tissues that have been monitored were fish muscles, gills and liver. Gills represent an important tissue for water pollution monitoring due to their direct contact with the water, while the liver has a high accumulation potential and as such represents an important pollution indicator (10, 11). Although muscles typically accumulate low or even minimal elemental concentrations, they often represent the major focus of toxicological research since they are the main part of the fish that is consumed by humans (12, 13). Other fish tissues that have a potential to be used as pollution indicators are gonads, intestines and kidneys.

Figure 1 presents general accumulation patterns of heavy metals and trace elements in muscles, gills and liver of fish from the Danube River basin, based on our previous research. It comprises the data for the Pontic shad (*Alosa immaculata*; 8), sterlet (*Acipenser ruthenus*; 9), barbel (*Barbus barbus*; 4), Prussian carp (*Carassius gibelio*; 7), silver carp (*Hypophthalmichthys molitrix*), bream (*Abramis brama*), white bream (*Blicca bjoerkna*), carp (*Cyprinus carpio*) and wels catfish (*Silurus glanis*; 14). As can be seen in the Figure, gills represent the center of the accumulation of Al, B, Ba, Mn and Sr, liver is the accumulation center of As, Cu and Mo, while both tissues have approximately equal frequency as accumulation centers of Fe and Zn. Muscle was most frequently the tissue with lowest elemental concentrations, with the exception of Sr and As, and partly Al, B, Ba and Cu. Future research within this field should include the assessment of additional fish tissues, of a wider spectra of fish species from various habitats and with differing life histories, as well as experimental approach that would establish relationship between elemental concentrations in the water and those in fish tissues.

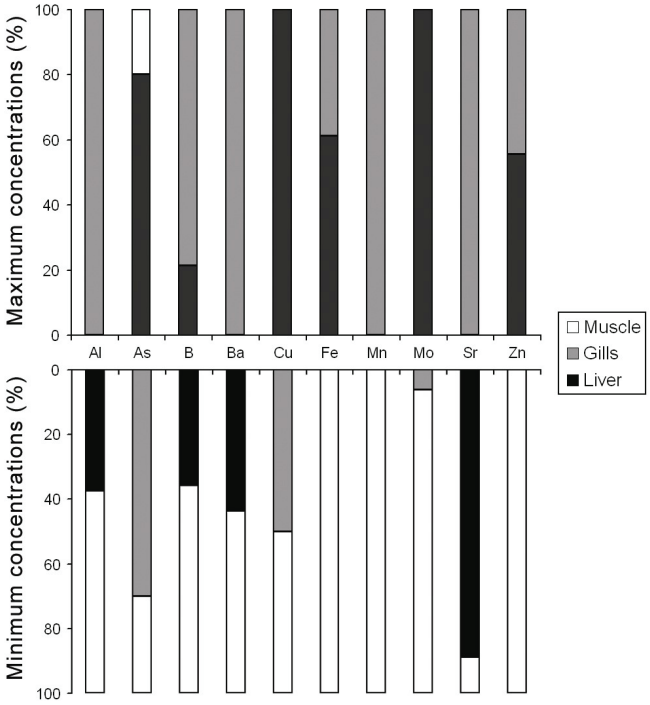

Figure 1: Ratio of the assessed fish species from the Danube River basin that had the maximum and minimum concentrations detected in a particular tissue, presented for each of the analyzed heavy metals and trace elements; data represent results from previous research (4, 7, 8, 9, 14)

Toxicological pathology

Toxicological pathology represents the study of the effects of pollutants on fish organs, tissues and cells. For the sample analysis, both light (LM) and electron microscopy (EM) can be used, although the use of light microscope is the most common approach for routine examinations and will be further discussed here.

Histological method procedure comprises sampling of fish organs, followed by immediate sample fixation (aldehyds or alcohols are commonly used LM fixatives) and dehydration in progressively higher ethanol concentrations. Following sample dehydration and clearing with xylene, samples are embedded in paraffin, sectioned using a microtome and stained with various histological stains (15). At the end of the protocol, the cells and tissues remain the same as they were at the moment of sampling, enabling examination and detection of tissues' alterations.

Advantages of the histopathological method lie in the fact that fish are located at the top of the trophic pyramid in the aquatic environment, which makes them suitable as markers of bioconcentration and biomagnification. Histopathology allows the assessment of target organs, namely those in immediate contact with the aquatic environment (i.e., gills and skin), as well as other affected vital organs (such as liver, gonads and kidneys). Histopathological analyses contribute to the understanding of pollutant activity mechanisms, or of the activity of a pollutant mixture. They also take into account specific sensitivity of various fish species to pollution, depending of their ecological niche or life history. Finally, since fish also represent a model of vertebrate organisms, detected effects of pollution can be extrapolated to mammals.

Histopathology is considered as a reliable bioindicator of aquatic pollution (16). Nevertheless, it also possesses certain drawbacks: histological changes are not pollutant specific; although the cost of the assessment procedure is relatively low, it requires specific equipment and trained staff; procedure is also time consuming, with considerable laboratory work needed for the preparation of samples; certain subjectivity of the method should be also emphasized, since

it depends substantially on the proficiency of the researcher; it is a descriptive method and quantitation is rather challenging. In their extended review, van der Oost et al. (17) discussed the relevance of histopathology as a biomarker of fish health status, giving it a score of 3.5 out of 5. The authors claimed that it is a relevant method, with high toxicological significance, but with a relatively low ability to detect the specific type of pollution. Other authors pointed out the advantage of histopathological approach in fish toxicology and ecotoxicology, particularly its usefulness in monitoring sublethal chronic effects of chemicals on fish (18), as well as its sensitivity to low levels of environmental contaminants (19).

However, it is not always easy to establish strong relationship between environmental pollution and histopathological alterations. Open water bodies are often exposed to mixtures of different chemicals, such as persistent organic pollutants, pesticides and endocrine-disrupting compounds, which produce diverse effects on fish tissues and organs (20). Moreover, while physical and chemical characteristics of water play an important role in the activation and fate of these substances (21), they can also impact organ morphology themselves (22). Other factors, such as the presence of heavy metals, parasites or cyanotoxins could produce negative effects on fish homeostasis and cause histopathological changes in various organs. Histopathological changes represent a general cumulative effect of all the stressors affecting the fish organism. Therefore, it is necessary to include more than a single organ in histological assessments, since some chemicals can induce changes only in a single organ, while the other organs of the same fish could remain unaffected.

Histopathology is subjective and often depends on the proficiency of a researcher. In the majority of manuscripts, an extent of a tissue alteration is usually categorized as either mild, moderate or a severe change (23), or as a percentage of the altered tissue (24). This can be avoided to an extent by using morphometric or stereological approaches (25, 26, 27, 28), which are, however, often time-consuming. Therefore, researchers are commonly assessing fish organs using semi-quantitative and/or scoring systems (29). Currently, we are using two scoring systems for histopathological analyses of fish tissues and organs. One of them is based solely on gill histology (30), while the second one takes into account four vital organs: gills, kidney, liver, and skin (1). Both systems are based on the same principle: besides the extent of detected alterations, there is also an importance factor of the type/severity of change, which multiplies the score. The sum of all detected changes is calculated separately for each organ, while the total index value represents a sum of scores for all assessed organs. The basic difference between two systems is the principle of describing the lesions: while Poleksic and Mitrovic-Tutundzic's system is taking into account specific gill changes (i.e. lifting of the epithelium, rupture and peeling of lamellar epithelium, telangiectasis, etc.), Bernet's system defines certain patterns of tissue response to pollution. Those reaction patterns are classified as: circulatory, regressive, progressive, inflammatory, and neoplastic, and each has subcategories, depending on the place where lesion occurs: epithelium or supporting tissue. This allows the researcher to use Bernet's scoring for different organs in the fish organism, because reaction patterns are universal to the tissues. The importance factor of both systems is in the same range: from 1 to 3, meaning that all lesions do not have the same importance/severity in the evaluation process. These scoring systems enable quantification and comparison between two or more polluted sites, or with the reference site. Scoring systems are commonly used in assessments of streams (31), rivers (32), lakes (33) and fish farms (34).

Genotoxicity testing

Water quality monitoring is frequently restricted to the measurement of physical and chemical parameters. However, established alarm thresholds for these parameters are related to toxic levels of polluting substances, and they usually do not take into consideration risks posed by chronic exposures at low pollutant concentrations, which are frequently present in complex mixtures and can produce genotoxic effects (35). Consequently, it is necessary to employ additional tests that are able to detect changes at the molecular level, especially those that can result in genetic mutations. Genotoxicity testing is considered as one of the most valuable fish biomarkers, and as such it should become an integral part within environmental risk assessments (17).

In recent years, the Comet assay and the micronucleus test have been increasingly used within ecogenotoxicology and for monitoring purposes. The main advantage of the Comet assay, when compared with the micronucleus test, is that it does not require the presence of cells that are undergoing mitosis, as well as its higher sensitivity to low concentrations of genotoxic substances. The Comet assay, also referred to as the single cell gel electrophoresis assay (SCG or SCGE), is a rapid, visual, and quantitative technique for measuring DNA damage in eukaryotic cells (36). Under alkaline conditions (pH >13), the assay is able to detect single and double-stranded breaks, incomplete repair sites, alkali-labile sites, and possibly also DNA– protein and DNA–DNA cross-links, in virtually any eukaryotic cell population that can be obtained as a single cell suspension. The Comet assay has found a wide application as a simple and sensitive method for evaluating DNA damage in fish exposed to various xenobiotics in the aquatic environment (37, 38). There are three different parameters used within the Comet assay to quantify the level of DNA damage: tail length, tail moment (Olive tail moment) and tail intensity (the percentage of the DNA located in tail). Olive tail moment is calculated as a product of two factors, the tail intensity and the distance between the intensity centroids (centers of gravity) of the head and the tail along the main axis of the comet. When using derived measurements (e.g. tail moment), data on primary measurements (i.e., tail length and tail intensity) should also be presented in the analyses (39). As there is a number of different parameters produced by this method, there is still a certain lack of consensus within the scientific community with regard to the most suitable and reliable Comet assay parameter. This could be resolved by employing other methods that would evaluate and compare parameters, and indicate those with the greatest sensitivity and reliability. One of such methods is the sum of ranking differences (SRD), which can be used to compare parameters and tissue combinations (40). In our previous study (41), we have applied SRD to evaluate nine different genotoxicity parameter/ tissue combinations - tail length, intensity and moment in three different cell types: erythrocytes, liver cells and gill cells. The study indicated that the Olive tail moment and tail intensity represent equally reliable parameters.

Nevertheless, Comet assay study design and data analysis still require further investigation, improvement and standardization. This is especially important for scoring methods (visual or computerized), DNA damage quantification parameters and experimental conditions, all of which intensely vary between laboratories (42). Although the Comet assay has a generally straightforward methodology, the image analysis is substantially more complex. Two approaches, visual analysis and computerized image analysis, are commonly used to measure DNA breakage in the Comet assay. In the visual analysis, comets are classified based on their morphology, either by grading their size or by measuring tail lengths (43), and the DNA damage is evaluated as an increase in the percentage of cells with comets (44, 45, 46). It is a relatively reliable and fast approach (47), but the results rely to a certain extent on subjective decisions made by the investigator. Computerized image analysis provides additional measurement criteria as compared with the visual approach, including those for tail length, moment and intensity. Since different laboratory conditions (such as incubation time, electrophoresis intensity and duration, gels and agarose concentration, etc.) can result in significantly different results, laboratories should conduct specific tests to obtain optimal resolution, and the parameters given above should always be specified as part of the experimental conditions of a Comet assay (48).

In our previous research (4, 41, 49), the Comet assay was employed to acquire information through the analysis of DNA damage *in vivo* in blood, liver and gills of European chub (*Squalius cephalus*) and in blood of barbel (*Barbus barbus*) from rivers and reservoirs in Serbia, which are under various degrees and types of pollution. Microscopic images of comets were scored using Comet IV Computer Software (Perceptive Instruments, UK). Gills gave the best response as compared to other tissues. Gills may be more prone to injury than other tissues, due to a high respiratory blood flow and permanent contact with the water environment. Blood was less sensitive in comparison to other tissues. This might be due to regular cycles of change of blood cells in the bloodstream, which indicates that blood could be used as a biomarker only for acute contaminations. Tissue specific responses are expected because of variations in alkali-labile sites and cell types with different background DNA single-strand break levels, due to variations in excision repair activity, metabolic

activity, antioxidant concentrations, or other factors (49). However, although the blood gave the lowest response to DNA damage compared to other tissues, it was still possible to observe the significant difference between the rivers with different intensity of anthropogenic influence (41). Our results confirmed that fish represent a good model system for genotoxicity testing within water pollution monitoring, as well as that evaluated genotoxicity biomarkers are sensitive and suitable for this type of research.

Biomathematics and statistics

Integrated use of different analytical techniques in water pollution monitoring commonly produces a large amount of data. Effective data analysis and the interpretation of results require specific data processing tools, such as chemometrics, which are able to provide optimization of the experimental design, data processing, clustering and pattern recognition, calibration, quality control and the organization of analytical processes. In our research we used principal component analysis (PCA) as a method suitable for data assessment and to illustrate group clustering (7), as well as canonical discriminant analysis (CDA) in cases when we had predefined groups to produce components, or variables, along which differences between groups are maximized while those within a group are minimized (3). Bearing in mind the known weaknesses of CDA for the evaluation of statistical analysis, post-hoc tests are also used as the most precise method for evaluating group differences (50).

Figure 2 presents a general approach of the integrated use of methods presented in this study, such as acquiring histopathological data and applying scoring systems, genotoxicity testing and measuring DNA damage, as well as detecting concentrations of different elements in fish tissues. Obtained results can be consequently used to distinguish fish species that have the greatest potential as aquatic pollution indicators. Future research should also include assessment of persistent organic pollutants in fish tissues and their pathological and genotoxic effects, enzymes involved in xenobiotic metabolism, blood parameters, as well as the use of discriminated functions and neural networks for data analysis. As a result, the general approach presented in Figure 2 could be enhanced by the inclusion of these methods and research topics.

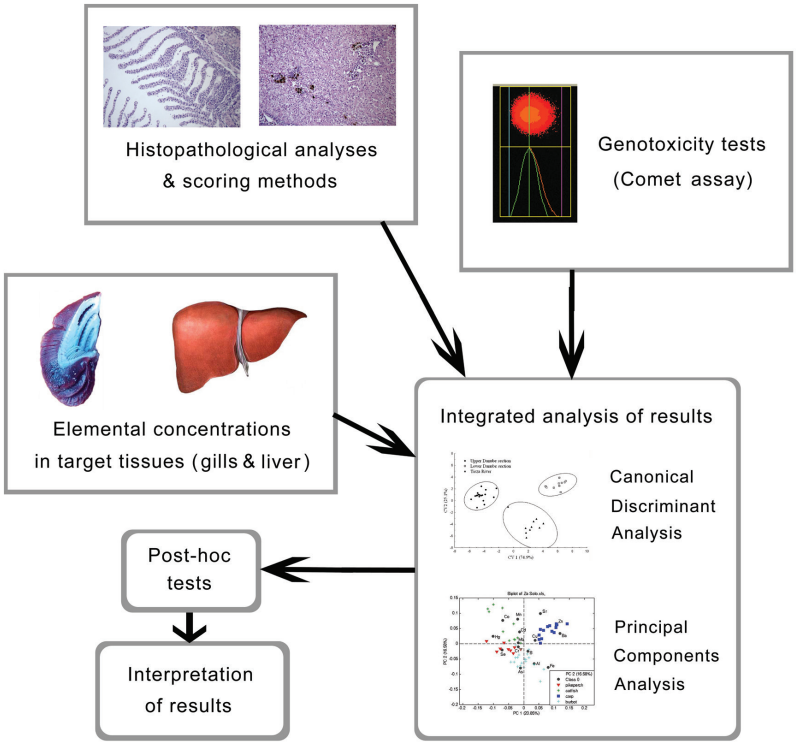

Figure 2: Schematic overview of the integrated use of different toxicological methods in aquatic pollution research and monitoring

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CELOSTNA UPORABA RAZLIČNIH RIBJIH PARAMETROV ZA OCENO STANJA VODA

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Povzetek : V sladke vode prehajajo visoke koncentracije različnih strupenih snovi. Ribe so običajno na vrhu vodne prehranjevalne verige, zato so primerni kazalniki onesnaženosti voda. Ocena prisotnosti toksinov in njihove strupenosti se lahko učinkovito izvede s kombinirano uporabo analizne kemije, bioloških testov in uporabne matematike. V tej raziskavi smo predstavili splošen pristop integrirane uporabe različnih parametrov, povezanih z ribami, za oceno onesnaženosti sladkovodnih voda. V članku predstavljamo lastne izkušnje s področja analitične toksikologije, toksikološke patologije in testiranja toksičnosti kot tudi biomatematičnih in statističnih metod, ki omogočajo integracijo rezultatov, pridobljenih s posameznimi metodami. Poleg tega je govor tudi o prednostih in pomanjkljivostih posameznih metod ter o potrebnih prihodnjih korakih pri njihovem razvoju. Ker se industrijske in komunalne odpadne vode v Srbiji še vedno ne očistijo, preden se spustijo v vodotoke, predstavljajo resno tveganje za vodne ekosisteme in zdravje ljudi. V članku opisani kazalniki onesnaženosti in parametri genotoksičnosti, ki predstavljajo pomembno orodje za učinkovito spremljanje vodnih ekosistemov. Metodološki pristop, predstavljen v članku, bo zanimiv za raziskovalce in menedžerje, ki se ukvarjajo z ekotoksikološkimi raziskavami in spremljanjem sladkovodnih ekosistemov

Ključne besede: sladkovodne ribe; težke kovine; histopatologija; genotoksičnost; PCA; CDA

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Original Scientific Article

ULTRASTRUCTURAL STUDY OF THE TRACHEA IN EXPERIMENTALLY INFECTED BROILERS WITH IBV SEROTYPE 4/91

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Summary: Avian infectious bronchitis virus (IBV) is a globally distributed Coronavirus that causes tremendous economic loss in the poultry industry. The primary target cells of the virus are located in the respiratory tract. Strain-dependent, IBV may spread to other epithelia and cause nephritis or drop in egg production. Subcellular changes in broilers trachea induced by infectious bronchitis virus serotype 4/91 were examined by transmission electron microscopy (TEM). Seventy 1-day-old commercial broiler chicks were divided randomly into two groups (control and experimental), and at the age of 21 days, all birds in the experimental group were challenged intranasally with the virus. Four birds from challenge group and two birds from the control group were euthanized at 1, 2, and 4 days post inoculation (PI) and tracheae were isolated after necropsy and examined with TEM. Mild tracheal rales, coughing and gasping were seen in the experimental group. As gross lesions, hyperemia and edema in tracheal mucosa were observed. IBV infection resulted in hypertrophy of goblet cells, their rupture, and the formation of excess mucus. At the level of the ciliated cells, complete deciliation of the tracheal surface was observed. Swelling and increase in the amount of endoplasmic reticulum was seen in infected birds.

Key words: transmission electron microscope; infectious bronchitis virus; serotype 4/91; trachea; broilers

Introduction

Avian infectious bronchitis virus (IBV), an enveloped, positive-sense single stranded RNA virus, is a member of the *Coronaviridae* family of the order *Nidovirales* that causes highly contagious disease of poultry, which poses a major threat to the poultry industry. The avian coronavirus IBV has a unique morphology. It is an envelope virus that is spherical to pleomorphic with evenly dispersed spike-like projections on the surface of the virion. Clinical cases of infectious bronchitis (IB) are associated with respiratory, reproductive, digestive and renal infections in domestic poultry and in various other avian species (1). Although effective vaccines are available and utilized routinely in commercial poultry production, the virus tends to mutate frequently (2). Little or no cross-protection occurs between different serotypes of infectious bronchitis virus (3), therefore, continuous determination of the epidemic serotype and production of new generations of vaccines are crucial for controlling IB in each geographic region or country. The IBV

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serotype 4/91 (also named 793/B and CR88), was first reported in Britain in the early 1990s (4, 5), and is one of the most common IBV serotypes worldwide (3). In Iran, the presence of Massachusetts serotype as the major circulating IBV was confirmed in 1994 (6). By isolation and serologic identification of some field strains, VASFI MARANDI and BOZORGMEHRI FARD (7), suggested the presence of IBV variant(s) in Iran. Serotype 4/91 was isolated in Iran by MOMAYEZ *et al.* (8) and a recent study revealed that it has been the dominant IBV serotype throughout 1994-2004 in Iran (9). In the present study, ultrastructural changes in the trachea due to infection with IBV serotype 4/91 were investigated.

Materials and methods

Virus preparation

IBV serotype 4/91 isolated from broiler flocks in Iran (10) was used in this study. Virus propagation was performed in 10-day-old embryonated chicken eggs. Eggs were obtained from a respiratory disease-free flock. The embryo lethal dose (ELD_{50}) of infected allantoic fluid was calculated according to the REED and MUENCH'S (11) formula. In the present study, allantoic fluid containing $10^{6.5}$ ELD_{50}/0.1ml of the virus was used to induce the disease.

Experimental Design

This study was performed after receiving approval from the Animal Ethics Committee of the Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran. Also, it was conducted with respect to the International Guidelines for research involving animals (Directive 2010/63/EU). Seventy, 1-day-old commercial broiler chicks (Ross breed) were divided randomly into two groups (fifty chicks in the experimental and twenty chicks in the control group). Experimental and control groups were housed in separate experimental rooms. Food and water were supplied ad libitum. No vaccine was used in either of the two groups. Prior to virus challenge, all birds were found free from IBV antibodies (Flock Check IBV ELISA test kit, IDEXX Laboratories Inc., Westbrook, ME). Tracheal swabs were taken from chicks for detection of other respiratory pathogens such as avian influenza virus (H9N2), Newcastle disease virus and *Mycoplasma spp.* by molecular assay before and after IBV inoculation (12) and the results were negative. At the age of 21 days, all birds in the experimental group were challenged intranasally with 0.2 ml of allantoic fluid virus suspension (titre $10^{6.5}$ EID50 per 0.1ml). Control birds were sham inoculated with an equal volume of normal saline. Four birds from the challenge group and two birds from the control group were euthanized with chloroform at 1, 2, and 4 days post inoculation (PI) and tracheae were isolated after necropsy.

Electron microscopy

Tracheal specimens were fixed in 2.5% gluteraldehyde–2% formaldehyde, washed in sodium cacodylate buffer solution (pH 7.4), post-fixed in 1% OsO4, dehydrated in ascending concentration of ethanol, and embedded in medium Spurr's resin. Tracheal sections (1 μm) stained with toluidine blue were examined for trimming of the blocks to areas of interest. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined in a Philips CM10 transmission electron microscope (13).

Results

Clinical signs and gross lesions

No clinical signs and gross lesions were observed in control group. In experimental group mild tracheal rales, coughing and gasping were seen at 24 hours PI. Severity of the signs was less after 5 days PI. Slight hyperemia and edema in tracheal mucosa were noticed in the euthanized birds. No mortality was observed in either group.

Electron Microscopy

In chicks from the control group the tracheal surface was lined by a pseudostratified columnar epithelium. The surfaces of control group tracheas were covered with abundant cilia and some goblet cells. The following changes were seen in chicks of experimental group at different days

post inoculation. In day 1 PI, virus particles were localized in the tracheal epithelial cells (Figure 1). In day 2 PI, limited infiltration of heterophils and lymphocytes to the tracheal epithelium, inflammation and congestion, hypertrophy of goblet cells, mild edema and partial loss of cilia were seen. In day 4 PI, deciliation of the tracheal epithelium was increased and complete deciliation was seen at some sections. Other changes in this time were: infiltration of plasma cells, and appearance of vacuoles containing lymphocytes and heterophiles (Figure 2), edema in sub mucosal layer, severe degeneration of the epithelial cells and deformation of the mucous gland. Viral particles were observed outside surface cytoplasmic membranes and occasionally within cytoplasmic vesicles (Figure 3). Swelling and increase in the amount of endoplasmic reticulum were seen at 4 PI (Figure 4).

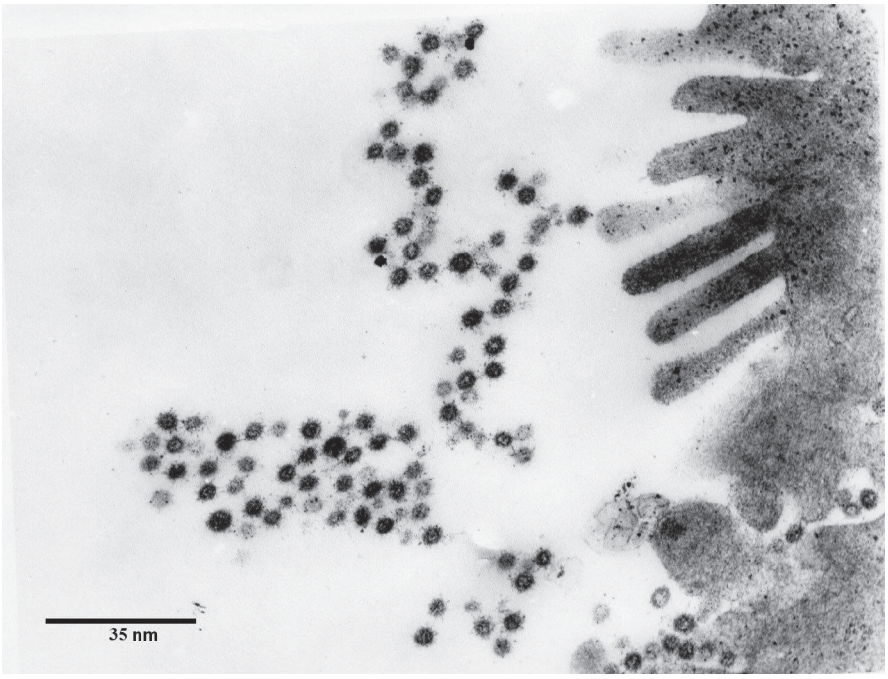

Figure 1: Viral particles in epithelial cells of chicken trachea at day 1 after infection. × 28500

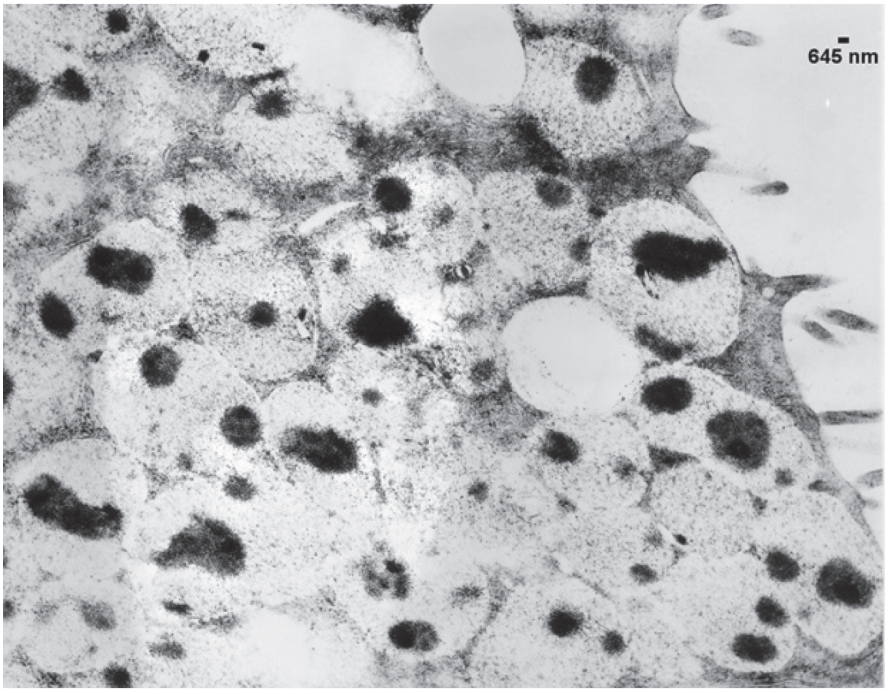

Figure 2: Trachea at 4 days post infection. Electron-dense particles in hypertrophied Goblet cells. Partial deciliation of the tracheal epithelium. × 1550

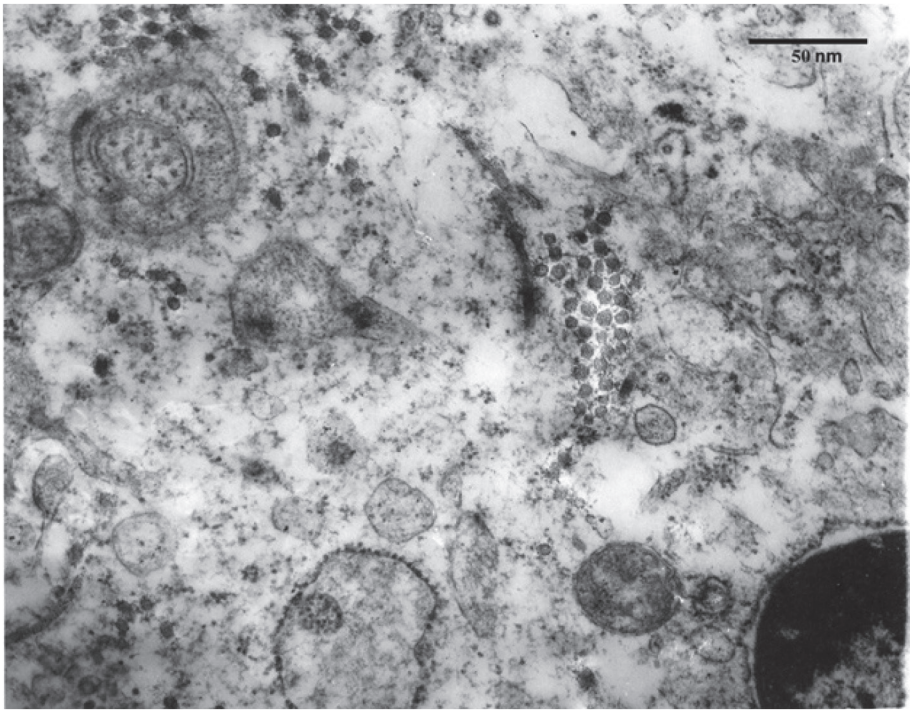

Figure 3: Viral particles are seen in the Golgi apparatus and in electron-dense areas at day 4 after infection. × 21000

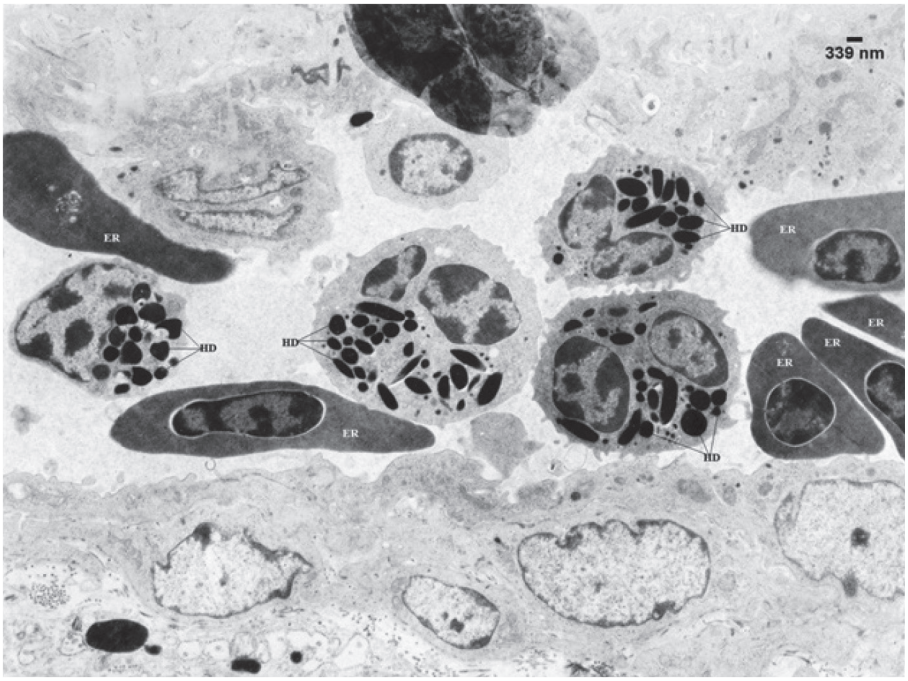

Figure 4: Heterophile degranulation at day 4 after infection. × 2950 (ER: Endoplasmic reticulum, HD: Heterophile degranulation)

Discussion

The progression of tracheal lesions induced by inoculation of commercial broiler chicks with infectious bronchitis virus serotype 4/91 was examined by tracking subcellular changes using transmission electron microscopy. Serotype 4/91 of IBV was first isolated in 1985 in France, and then spread to many countries in Europe, Japan, Saudi Arabia, Thailand and Mexico (5, 14). Despite the regular vaccinations on chicken farms, mostly with Massachusetts (Mass) strains, IB still exerts a severe negative impact on the poultry industry in Iran. Cross-protection between different serotypes of IBV is variable; hence vaccination failure may be due to low homology (26%) between the 4/91 and Massachusetts serotype vaccines such as the H120 strain (15, 16). Therefore, it is very important to understand the effects of this virus on broilers.

Replication of IBV occurs in the ciliated epithelium and mucous cells within 24 hr after intratracheal or aerosol inoculation, and viral particles are confined to small vacuoles of cytoplasm (17, 18). Severity of the tracheal lesions are varied due to different inoculation routes, strains and inoculum sizes of IBV and age of chicken infected (17, 19, 20). In our experiment, tracheal epithelial changes such as hyperemia and edema were similar to those described previously (21, 22).

According to the results, complete deciliation was seen at 4 PI, which is in agreement with results of UPPAL and CHU (23), NAKAMURA *et al.* (20), and ABD EL RAHMAN *et al.* (24). Loss of cilia was observed at day 2 PI and was complete at day 4 PI. The increase in the damage to ciliated cells destruction between 1 and 4 days PI reflects the replacement of the highly differentiated, pseudostratified epithelium containing ciliated cells by a simple squamous to cuboidal epithelium without cilia. Replacement by cuboidal cells is best interpreted as the regeneration phase during which the basal cells at the periphery of a lesion slide across the underlying fibroblastic connective tissue to close the lesion. An early strong activation of the goblet cells was observed in inoculated chicks which most likely reveal the initial IBV infection of these cells. The subsequent decrease in the goblet cells activation score can be attributed to the goblet cells exhaustion and elimination due to virus replication as severe ultrastructural decay and rupture of these cells was observed after the initial activation. Cilia are responsible for propelling the entrapped particles (bacteria, virus, dust etc.) for disposal. Reduced ciliary motility or disrupted ciliated epithelium could be expected to adversely affect the resistance of birds to microorganisms that normally enter their bodies via the respiratory system (25). On the other hand, this study confirmed that IBV initially infects the upper respiratory tract, where it is restricted to the ciliated and mucus-secreting cells (26).

In Iran, the most prominent lesion in respiratory disease infected flocks is severe exudation in trachea, which leads to tubular cast formation in the tracheal bifurcation and extending to the lower bronchi (27). This is followed by destruction of the cilia by factors such as infectious bronchitis virus.

In normal conditions in which the goblet cells are not activated, the mucus layer that covers the tracheal epithelium is largely washed away during the reparative steps. After virus inoculation goblet cells were ruptured and deformed. This finding is in line with results of MAST *et al.* (28).

In conclusion, although no mortality was seen in infected group in experimental condition, IBV serotype 4/91 can increase the susceptibility to the respiratory disorders by tracheal hyperemia and deciliation in field status.

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ULTRASTRUKTURNA RAZISKAVA SAPNIKA V POSKUSNO OKUŽENIH BROJLERJEIH Z IBV SEROTIPA 4/91

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Povzetek: Aviarni virus kužnega bronhitisa (IBV) je globalno razširjen korona virus, ki povzroča ogromno gospodarsko škodo v perutninski proizvodnji. Primarne tarčne celice virusa so v dihalnem traktu. IBV se lahko razširi tudi na druga epitelijska tkiva in povzroči npr. vnetje ledvic ali padec proizvodnje jajc.
S transmisijsko elektronsko mikroskopijo (TEM) smo pregledali znotrajcelične spremembe sapnika brojlerjev po okužbi z virusom kužnega bronhitisa serotipa 4/91. Sedemdeset enodnevnih brojlerskih piščancev je bilo naključno razdeljenih v dve skupini (kontrolna in poskusna). Pri starosti 21 dni so bili vsi piščanci v poskusni skupini okuženi z virusom preko nosu. Štiri piščance iz poskusne skupine in po dva iz kontrolne smo usmrtili v 3 časovnih točkah po okužbi, natančneje po enem dnevu, po dveh in po štirih dneh. Ob obdukciji smo izolirali sapnike in jih pregledali s TEM. V poskusni skupini smo opazili blago obliko hropenja, kašelj in zasoplost. Močneje sta bila izražena hiperemija in edem sluznice sapnika. Okužba z IBV je povzročila hipertrofijo celic goblet, njihovo pokanje in prekomerno tvorbo sluzi. Na ravni ciliarnih celic smo opazili popolno odsotnost migetalk na površini sapnika. Pri okuženih piščancih smo opazili tudi otekel in povečan endoplazemski retikulum.

Ključne besede: transmisijski elektronski mikroskop; virus kužnega bronhitisa; serotip 4/91; sapnik; brojlerji

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Original Scientific Article

THE USEFULNESS OF TWO MOLECULAR METHODS FOR THE DETECTION OF PERSISTENTLY INFECTED CATTLE WITH BOVINE VIRAL DIARRHEA VIRUS USING ORAL SWAB SAMPLES

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Summary: Serum and oral swab samples were collected from a persistently infected cow and her calf in a two-month period to test usefulness of oral swab samples for the detection of bovine viral diarrhea virus. Nucleic acids of the virus were detected by two molecular methods: conventional gel-based RT-PCR and commercial real-time RT-PCR. The bovine viral diarrhea virus genome was detected in serum and oral swab samples on days 0, 7, 15, 22, 23, 29, 36, 37, 43, 44, 46, 51, 52, 53, and 57. The dry cotton swabs showed a reduction of diagnostic sensitivity after three days when samples were stored at room temperature (+ 21 °C), but storage of oral swab samples at + 4 °C or in a freezer (< – 15 °C) for at least 10 days had no negative impact on the detection of the virus. No reduction of diagnostic sensitivity was observed when oral swab samples were collected in tubes with a liquid virus transport medium. Oral swabs provide an easy, reliable and cost-effective sampling tool for identification of PI animals, together with RT-PCR methods. The oral swab sampling could be especially useful for screening newborn calves during testing and removing PI animals from bovine viral diarrhea virus-infected herds.

Key words: bovine viral diarrhea virus; diagnostics; oral swab; RT-PCR; real-time RT-PCR

Introduction

Bovine viral diarrhea (BVD) is a disease of cattle that reduces productivity and may increases death loss. It is caused by bovine viral diarrhea virus (BVDV), a member of the *Pestivirus* genus of the family *Flaviviridae* (1). BVDV is distributed throughout the world, with endemic areas detecting antibodies among 70%–100% of herds, while in some European countries such as Sweden, Norway, Finland and Austria, the disease has already been eradicated (2). BVDV is spread by close contact (nose-to-nose) between cattle. Virus is shed by both acutely and persistently infected (PI) animals, but levels of shedding are much higher in persistently infected cattle, which are the natural reservoir for the virus. Fetuses that become infected between 30 and 125 days of gestation and survive the infection may be born as BVDV-infected calves. The BVDV infection will persist for the life of the calf, hence the term "persistent infection", or PI. It is estimated that the incidence of PI animals is between 0.3 and

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2.6% (3, 4). PI animals are the main source of infection in infected herds and tend never to reach their productive potential and growth because of reduced fertility and increased susceptibility to other diseases (4). PI animals shed large amounts of virus in all their secretions and excretions (5). By removing PIs from the population, the source of infection is removed, and by this means, the disease can be controlled (2). Blood tests are the most frequently used method to identify BVDV in live animals. Tests can also be done on skin biopsies (taken from the ear), on milk or even on hair samples (2, 5, 6). The oral swab sampling method has become more important both in human and veterinary fields because it is less uncomfortable for the animal during sampling. It is also a simple method for farmers and can be performed without special technique or equipment (7, 8, 9, 10, 11, 12, 13). To date, only one research paper describes the successful testing of oral swab samples as an option for the detection of PI animals from BVDV-infected herds (14).

The purpose of this study was to use two RT-PCR tests for BVDV on PI cattle over a two-month period to (a) determine the usefulness of oral swab samples for the detection of BVDV in PI animals, (b) determine the sensitivity of two molecular methods for detection of BVDV from oral swab samples and (c) test the robustness of two types of swabs for oral sampling during 10 days of storage at three different temperatures.

Materials and methods

A persistently infected pregnant cow, 58 months old, was identified in a BVDV-infected herd and removed to another location where all samplings were carried out. Serum and oral swab samples were collected from the PI cow at days 0, 7, 15, 22, 29, 36, 43, 51 and 57. A clinically healthy calf was born on day 22 after the first sampling, and then serum and swab samples were also collected from the calf on days 22, 23, 29, 36, 37, 43, 44, 46, 51, 52, 53 and 57, after the first sampling in the cow. Oral swab samples containing saliva from the oral cavity of the cow and calf were collected using sterile dry cotton swabs and were immediately sent to a laboratory. The samples were homogenized in 1 ml of RPMI 1640 cell culture medium (Gibco, United Kingdom) and stored in a freezer at < – 15 °C until testing. To test the stability of viral RNA in a dry cotton swab stored at three different temperatures, 3 x 11 oral swab samples were collected on day 57 from the cow, and then 11 samples were placed for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days at room temperature (18–24 °C), 11 samples for 0 to 10 days in a refrigerator (5 ± 3 °C) and 11 samples for 0 to 10 days in a freezer (< – 15 °C). After storage at different temperatures, the oral cotton swab samples were homogenized in 1 ml of RPMI 1640 cell culture medium (Gibco, United Kingdom) and stored < – 15 °C until testing. Additional 3 x 11 oral swab samples were collected from the calf on day 57 using Virocult® tubes with liquid virus transport medium (MWE, United Kingdom) and were then stored at three different temperatures as described above.

Total RNA was extracted from 140 µl of homogenate or the liquid with Virocult® transport medium (MWE, United Kingdom) using a commercial kit for RNA extraction QIAamp® Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. One-step RT-PCR was performed on samples in one tube using primer sequences based on 5' non coding region (5'NCR), with the forward primer P 104F 5'-GCT AGC CAT GCC CTT AGT AGG ACT-3' and the reverse primer P 402R 5'-CAA CTC CAT GTG CAA TGT ACA GCA-3', which detect both genotype 1 and 2 strains of BVDV (15). The reference BVDV strain NADL was used as the positive control. Reaction mixtures without RNA served as negative controls. The reaction was performed in a total volume of 25 µl using One-Step RT-PCR® Kit (Qiagen, Germany) as follows: 15 µl of nuclease free water, 5 µl of 5x PCR buffer, 1 µl of dNTP mix (containing 10 mM of each dNTP), 0.5 µl of the stock solution with 20 µM of each primer, 1 µl of the one-step RT-PCR enzyme mix and 2 µl of the RNA template. The RT-PCR program included a reverse transcription stage at 50 °C for 30 min, followed by an initial PCR activation step at 95 °C for 15 min. This was followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min and a final extension step at 72 °C for 10 min. The reactions were carried out on a programmable T1 thermocycler (Biometra, USA) and the RT-PCR products were visualized in 1.8% agarose gel with 0.5 µg/ml ethidium bromide, with subsequent visualization under a UV light. The size of the PCR product was compared to the 100 bp DNA ladder (Fermentas, Germany) and PCR products of about 300 bp were interpreted as positive according to

the expected DNA fragment and as negative if no specific product was visible.

Serum and oral swab samples were quantified using a commercial real-time method (RT-qPCR) TaqVet® BVDV Screening (Laboratoire Service International, France) for the detection of BVDV. After RNA extraction, RT-qPCR was performed on Mx3005P thermocycler (Stratagene, USA) using protocol according to the manufacturer's instructions, and the results were presented as a cycle threshold value for individual samples. Analysis of RT-qPCR amplification curves was performed using commercial thermal cycler system software, and an "auto baseline" was used to determine fluorescence baselines. All samples were tested in one run on the same 96-tube microplate.

The results of statistical evaluation are shown as an average ± standard deviation of the average (SD). In order to test the differences in each parameter among time sampling, t-test for two paired samples / two-tailed test and the Pearson correlation coefficient were used. For assessing agreement between two methods of clinical measurement, Bland-Altman methods were used (16). SigmaStat 3.5 (SYSTAT Software Inc.) and XLSTAT 2013 (Addinsoft 1995–2013) software was used.

Results

During the 57 days of the study, in total, 21 serum and 21 oral swab samples were collected from the two PI and tested by RT-PCR and RT-qPCR methods (Table 1, Table 2). Viral RNA was detected in all serum and oral swab samples by both methods, confirming 100% successful detection of PI animals in both types of samples (Figure 1). The detected Ct value in serum samples varied between 20.34 and 24.89 (average ± 2 x Standard Deviation: 22.88 ± 2.54), while the detected Ct value in oral swab samples was between 21.74 and 29.97 (26.26 ± 4.42); the differences between values were statistically significant ($P<0.001$). About a 3.4 lower Ct value for oral swab samples was expected compared to detected Ct value in serum samples, because during preparation of oral cotton swab samples, the dilution 1:10 of swab samples in cell culture medium was used (Figure 2). The coefficient of variation (CV) for serum samples was 5.54 and for oral swab samples, 8.40. No significant differences were observed between Ct values obtained from cow and calf (cow-serum: 23.51 ± 1.34; cow-oral swab: 26.63 ± 3.76; calf-serum: 22.40 ± 2.84; calf-oral swab: 26.16 ± 5.0) during the time of the study.

Table 1: Detection of RT-PCR product on gel electrophoresis by using RT-PCR method for the detection BVDV after storage of oral swab samples at room temperature (+ 21 °C), in refrigerator (+ 4 °C) and in freezer (< – 15 °C) for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days

	OS-C (+ 21 °C)	OS-C (+ 4 °C)	OS-C (<- 15 °C)**	OS-V (+ 21 °C)	OS-V (+ 4 °C)	OS-V (<- 15 °C)
day 0	+	+	+ **	+	+	+
day 1	+	+	+ **	+	+	+
day 2	+	+	+ **	+	+	+
day 3	+	+	+ **	+	+	+
day 4	-	+	+ **	+	+	+
day 5	-	+	+ **	+	+	+
day 6	-	+	+ **	+	+	+
day 7	-	+	+ **	+	+	+
day 8	-	+	+ **	+	+	+
day 9	-	+	+ **	+	+	+
day 10	-	+	+ **	+	+	+

*Two types of oral swabs were used: oral cotton swab samples (OS-C) and oral swab samples using Virocult® tubes with liquid virus transport medium (OS-V). The results in the third column OS-C (< -15 °C)** are for stored samples in the freezer (for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 weeks). Results are presented as positive (+) when RT-PCR product was detected and as negative (-) when RT-PCR product was not detected.

Table 2: Detection of BVDV by using RT-qPCR method after storage of oral swab samples at room temperature (+ 21 °C), in refrigerator (+ 4 °C) and in freezer (< – 15 °C) for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days

Days	OS-C (+ 21 °C)	OS-C (+ 4 °C)	OS-C (< - 15 °C)**	OS-V (+ 21 °C)	OS-V (+ 4 °C)	OS-V (< - 15 °C)
day 0	29.85	24.87	27.46**	24.92	23.91	25.16
day 1	31.55	26.69	30.08**	23.86	25.46	25.36
day 2	34.14	28.23	27.12**	30.29	25.89	26.35
day 3	38.26	27.32	30.27**	28.47	27.21	25.93
day 4	>45	26.93	33.49**	32.24	26.16	24.9
day 5	>45	27.35	32.47**	26.4	25.96	26.22
day 6	>45	31.68	32.39**	32.2	32.55	25.6
day 7	>45	31.02	31**	32.97	27.19	23.93
day 8	>45	30.01	34.32**	27.9	26.57	23.29
day 9	>45	28.79	29.21**	30.58	28.2	26.8
day 10	>45	30.42	32.75**	29.45	28.86	26.53
X	Not done	**28.48**	**30.96**	**29.03**	**27.09**	**25.46**
SD	Not done	2.10	2.38	3.04	2.25	1.10
X+2xSD	Not done	32.69	35.72	35.10	31.60	27.65
X-2xSD	Not done	24.28	26.20	22.95	22.58	23.27
CV	Not done	7.38	7.68	10.46	8.32	4.30

*Two types of oral swabs were used: oral cotton swab samples (OS-C) and oral swab samples using Virocult® tubes with liquid virus transport medium (OS-V). Results are presented as positive (with Ct value of each sample) and negative (with Ct value > 45) when Ct value was not detected. The results in the third column OS-C (< -15 °C)** are for samples stored in the freezer for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 weeks.

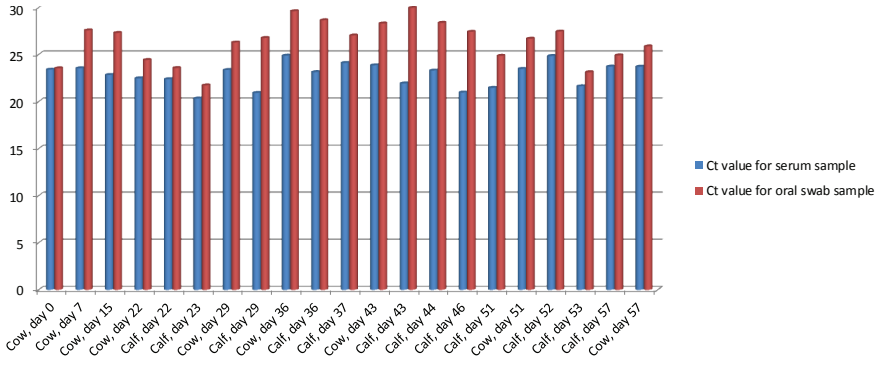

Figure 1: Detection of BVDV RNA in serum and oral cotton swab samples from PI cow and calf during 57 days of study. The results are presented with cycle threshold values (Ct) obtained by commercial real-time method for each day of sampling

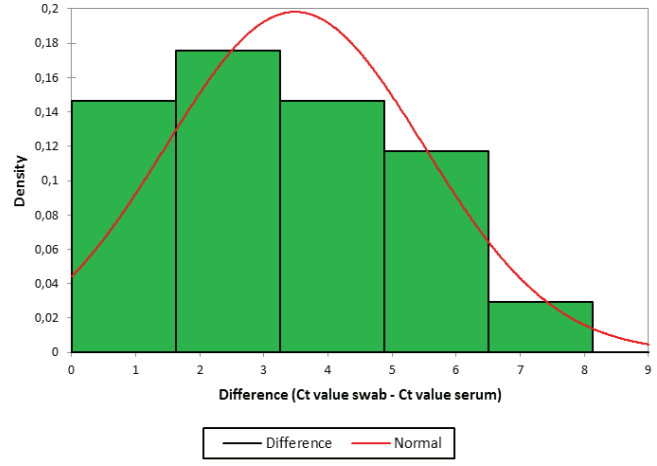

Figure 2: Plot of the difference between the Ct values obtained for 21 swabs and serum samples (average difference of Ct was 3.484)

Additional oral cotton swab and Virocult® swab sampling was used, and samples were stored at three different temperatures to test the stability of viral RNA for 10 days. When comparing the results of gel-based RT-PCR and commercial RT-qPCR, excellent matching between both methods was observed. The degradation of viral RNA in cotton oral swab samples was observed when oral cotton samples were stored at room temperature, and after four days, a negative result was detected in both methods. The degradation of viral RNA was evident through Ct values obtained by the RT-qPCR method (day 0 Ct = 29.85; day 1 Ct = 31.55; day 2 Ct = 34.14; day 3 Ct = 38.26; days 4–10 Ct = > 45). When using Virocult® swab sampling, all samples were detected positive by both methods after storage of the samples 10 days at room temperature. The storage of both types of oral swab samples for 10 days in a refrigerator (+ 4 °C) or in a freezer (< – 15 °C) had no influence on detection of BVDV in samples by RT-PCR methods. According to the results of this experiment, the storage condition of oral swab samples can influence detection of BVDV, but in general, keeping oral swab samples at + 4 °C or in a freezer (< – 15 °C) for 10 days after sampling has no impact on the sensitivity of gel-based RT-PCR and RT-qPCR. Additional testing was done on the stability of oral swab samples stored in the freezer (< – 15 °C) for 10 weeks, with detection of BVDV in all samples (average Ct = 30.96 ± 4.76 and CV 7.68; individual results are presented in Table 2). According to the uniform Ct values (Ct between 31.39 and 34.52, CV=1.69) for internal control RNA by RT-qPCR, no presence of inhibitors was detected in any of the 66 tested oral swab samples.

Discussion

The fluid collected by oral swabs is composed of saliva in the buccal cavity, produced by the salivary gland, and transudate that originates from the circulatory system; thus our results support the idea that oral samples can successfully replace some other types of samples. The general observation of this study is that the results of two molecular methods for the detection of BVDV in oral swab samples, presented equal sensitivity with comparison to results for serum samples. BVDV was detectable in the PI cow and calf for 57 days in all serum and oral swab samples tested by gel-based RT-PCR and commercial RT-qPCR. The results of the two molecular methods are comparable, despite the relatively large bias value, confirming that both molecular methods were suitable for testing oral swab samples. The structure of our study was different from previously published (14) although the data support the observation of Tajima and co-authors. According to detected Ct values in both PI animals during study, it was confirmed that the quantity of viral RNA detected by RT-qPCR in oral swab samples (saliva) was very similar to the quantity detected in serum (Ct values) collected on the same day. As shown in Figure 1, the relative quantities of viral genome variation exist from serum to serum and also from oral swab samples. Our observation in oral swab samples supports the data for detection of BVDV in nasal secretions and ear-notch samples from PI animals during a longer period (2, 5). Variations in Ct value which were observed for oral swab samples compared to serum samples are probably a result of vigorous or mild sampling of saliva in the oral cavity by different operators, but these variations were far from producing false negative results. Although only few data has been published to date regarding the usefulness of oral swab sampling for BVDV detection in PI animals, different data has also been presented for diagnostic sensitivity for other type of samples. A previous study, comparing eight types of samples, including oral swabs, collected from 40 PI animals, showed that oral swab samples were detected as BVDV positive by antigen capture enzyme-linked immunosorbent assay (ACE) in only 8% of samples (17). This observation is contrary to the results of our study and suggests a strong influence of the diagnostic method on the final results. Our study also confirmed, contrary to previous observation, that the viral load in oral fluid is very similar to that in serum. Different types of ACE are widely used in many laboratories to detect viral antigens in either serum or tissue samples, but according to previously observation ACE is not suitable for testing saliva samples. When using ACE, the target molecule is viral antigen, while when using RT-PCR, the target molecule is viral nucleic acid. The influence of the storage of samples on contamination with enzymes and inhibitors may be the reason for the low sensitivity of AEC when testing oral samples. Interestingly, in the same study, the nasal swab samples showed 100% sensitivity with AEC (17). The storage conditions during transport of any type of samples into the

laboratory, not to mention the time of transport, can vary from sample to sample and can influence the results. For this reason, two types of oral swabs were stored three different temperatures, and then oral swab samples were evaluated for 10 days to test their robustness during transport. The results of our study confirmed that storage of oral samples at room temperature (18–24 °C) reduced the sensitivity of molecular methods for BVDV detection from oral cotton swabs after four days, but when using Virocult® tubes with liquid virus transport medium for oral swab sampling, the sensitivity was not affected during the 10 days (Table 1, Table 2). The sensitivity of the two molecular methods was not reduced for samples stored at + 4 °C or at < – 15 °C for at least 10 days. In addition, all 11 samples were detected as positive during 10 weeks of storage at < – 15 °C. These data suggest that oral cotton swabs can be useful for sampling, but for this type of samples, it is recommended that they are stored at + 4 °C during transport. For oral sampling, similar to nasal swabs, tubes with liquid virus transport medium will be recommended, because this medium will also stabilize the viral RNA in samples at room temperature. In addition, when arriving in the laboratory, such samples do not need homogenization, which is practical for skipping the process of dilution of oral samples, thereby not losing the sensitivity because of that process. This would be especially important when the laboratory pools individual samples prior to extraction of RNA. The important finding in this study is that oral swab samples are useful when detection of BVDV is done by gel-based RT-PCR and RT-qPCR.

The current study revealed that oral swab samples have an equal sensitivity for the detection of PI animals compared to the standard serum samples. Oral swab sampling offers an efficient, easy, cost-effective solution for farmers, during an eradication program, to identify PI animals in infected herds. This type of sample could be especially useful for removing PI animals from an infected herd and for searching for PI animals among newborn calves for 9 months after the removal of the last PI animal. Early testing of newborn calves during the following 9 months is crucial for success, because it reduces the possibility that a newborn PI calf is the source of possible new infections (2). If serum sampling is used, this requires a call to a veterinarian for each newborn calf on the farm, but an oral swab sample can be taken and processed by an individual farmer. Oral swab samples can be a good alternative to the widely used ear-notch and serum samples. Of course, high quality identification of individual animals and careful record keeping is, in any case, very important for tracing the data.

The molecular method has been widely accepted in recent years because of its rapid turnaround time, its possibility for quantification and the fact that it enables the testing of a pool of 25–50 individual samples to reduce costs (14, 18). Although laboratory pooling of oral swab samples was not tested in our study, the observations from our study provide evidence for the possibility of the pooling of samples that is similar to pooling for serum or ear-notch testing.

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UPORABNOST DVEH MOLEKULARNIH METOD ZA ODKRIVANJE TRAJNO OKUŽENIH ŽIVALI Z VIRUSOM BOVINE VIRUSNE DIAREJE NA VZORCIH USTNEGA BRISA

I. Toplak, D. Rihtarič, P. Hostnik, J. Mrkun

Povzetek: Dva meseca smo pri trajno okuženi kravi z virusom bovine virusne diareje (BVD) in njenem teletu vzporedno odvzemali vzorce serumov in ustnih brisov, da bi testirali uporabnost vzorcev sline za dokazovanje prisotnosti virusa. Nukleinsko kislino virusa smo dokazovali z dvema molekularnima metodama: s klasično RT - PCR z elektroforezo v agaroznem gelu in komercialno metodo RT - PCR v realnem času. Genom virusa BVD smo dokazali v vseh vzorcih serumov in ustnih brisov, odvzetih na 0, 7. 15. 22. 23. 29. 36. 37. 43. 44. 46. 51. 52. 53. in 57. dan od začetka vzorčenja. Pri odvzemu vzorcev brisov s suho bombažno vatenko in po tridnevnem hranjenju vzorca na sobni temperaturi (+ 21 °C) smo ugotovili zmanjšanje diagnostične občutljivosti. Ko pa smo vzorce ustnih brisov hranili do 10 dni pri + 4 °C ali v zamrzovalniku na manj kot minus 15 °C, pa takšno hranjenje ni imelo negativnega vpliva na dokazovanje virusa. Znižanja diagnostične občutljivosti pri brisih pa nismo ugotovili, ko smo za odvzem vzorcev uporabili komplet komercialnega brisa, ki vsebuje transportno gojišče. Ustni bris omogoča enostavno, zanesljivo, učinkovito in cenejše vzorčenje pri identifikaciji trajno okuženih živali in zanesljivo diagnostiko, skupaj z uporabo metode RT - PCR. Uporaba vzorcev ustnih brisov bi lahko bila še posebej priročna pri pregledu novorojenih telet in odstranjevanju izločevalcev virusa iz govejih čred, ki so okužene z virusom BVD.

Ključne besede: virus bovine virusne diareje; diagnostika; ustni bris; RT-PCR, RT-PCR v realnem času

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Original Scientific Article

TREATMENT OF ACARIASIS WITH IVERMECTIN AND EVALUATION OF DIFFERENT SAMPLING TECHNIQUES IN MICE

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Summary: Breeding mice positive for *Myocoptes musculinus* and *Myobia musculi* were treated with ivermectin in doses of 200 µg/kg of body weight (6µl of active substance) as a spot application on the back of the neck. Application was repeated three times in seven-day intervals. Different development forms (adults, nymphs, larvae, and eggs) were observed in groups of adult and young mice before the treatment as well as during and after the treatment.

Before treatment, the fur pluck technique and sticky paper technique (sampling from two different places: from back and abdomen) were evaluated. The fur pluck technique was used as the gold standard. The sticky paper technique has 91.5 to 93.2 % sensitivity for Myocoptes musculinus and 10.8 to 13.5 % for *Myobia musculi*. We observed that *Myobia musculi* place eggs next to the skin, on the bases of two or more hairs together; consequently, the sampling is not always satisfactory.

The effectiveness of therapy with ivermectin for *Myocoptes musculinus* and *Myobia musculi* was shown after the second treatment. Before the treatment, as well as during and after the treatment, a significantly higher percentage of positive mice was observed among old than in young ones. After the third treatment, adults and developmental stages (eggs, larvae, and nymphs) were still found only in Myocoptes musculinus. Almost all eggs of *Myocoptes musculinus* and *Myobia musculi* were drained and damaged after the third treatment.

Key words: veterinary science; mice; ectoparasites; *Myocoptes musculinus*; *Myobia musculi*; treatment

Introduction

Fur mites are a persistent problem in mouse colonies, especially in crowded laboratory colonies or in animals in poor condition (1). Murine acariasis is frequently caused by *Myocoptes musculinus*, *Myobia musculi* and *Radfordia affinis* (2, 3). Fur mites are primarily transmitted through direct mouse-to-mouse contact (1). The most frequently published antiparasitic drugs for the treatment of mites in rodents are ivermectin (4, 5, 6, 7, 8), selamectin (9, 10) and moxidectin (10). However, since the 1990s, mite infections have been controlled with different acaricides, including neguvon (4, 8), dichlorvos, permethrin, pyrethrins and trichlorfon (9).

Most protocols describing effective fur mite eradication require individual treatment of animals (9) or the preparation of medicated water bottles (4, 11). Arbona et al. (6, 7) evaluated the administration of an oral acaricide in feed by using ivermectin-compounded feed in a large colony with over 120,000 mice. Such treatment was very useful for large colonies but for small colonies individual treatment is more frequently

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used. Metcalf Pate et al. (3) reported different successful eradication of fur mites with topical parasiticides, oral drugs, ivermectin-impregnated feed, injectable ivermectin, environmental agents and cross-fostering paired with ivermectin treatment. Gonenc et al. (9) emphasized that the choice of acaricide, method of application and treatment interval are essential in the control of these ectoparasites.

The degree of pathogenicity to mice is variable among strains and even in single colonies (9). Fur mites can cause clinical signs in infested mice, which include localized pruritus, alopecia, ulcerative dermatitis, lymphadenopathy, weight loss (2), auto-traumatization and secondary amyloidosis (12).

The identification and eradication of murine fur mite infestations are ongoing challenges faced by many research institutions (2) and other mouse-breeding facilities, including pet shops. Roble et al. (13) investigated the prevalence of ectoparasites, endoparasites and infectious agents carried by apparently healthy mice obtained from pet stores.

In our research, a mouse breed intended for pet stores or as a reptile food was treated for mites. The most commonly used concentration of ivermectin (200 µg/kg body weight (BW)) (5, 6) was used. Three different techniques were used, and the results had been compared between fur pluck technique, sticky paper technique from the back and sticky paper technique from the abdomen. The fur pluck technique was the most sensitive technique; therefore, it was used to monitor the effect of treatment with ivermectin.

Material and methods

Animals

A colony of white mice consisted of six large terrariums with a group of 500 mice in each (reproductive units). The ratio of bucks and does in boxes is about 1:20 in favour of females. A few days before giving birth, mice are moved into small terrariums (nursery units). Ten to twenty females reside together with their baby mice until their removal at the age of 20 days (units intended for sale).

A group of 59 mice in nursery units were treated with Ivermectin 10, solution for injection (1 % ivermectin – 10 mg/ml – 1 % w/v). From twelve nursery units three, with 10, 14 and 15 does were chosen randomly. Baby mice involved in the investigation originated only from one unit with 10 mice. Ivermectin was diluted 1:50 with a mixture of equal amounts of propylene glycol and water to obtain a 0.02 % concentration of the active ingredient (200 µg/kg BW). Treatment of 39 does (30 g BW), and 20 young mice, three weeks old (10 g BW) was performed. Does were given 30µl diluted preparation and young mice 10 µl. The Ivermectin solution was placed as a spot application on the back of the neck. Application was repeated three times in seven-day intervals. Pipette and tips (Biohit pipette 5–25 µl) were used for the precise dosing of the drug. These animals were monitored individually throughout the experiment: before, during and after the treatment.

Sampling procedure

Before treatment, the samples of fur were taken in different ways, from different places, and divided into three groups. Fur was collected via the impression of 3 cm long duct tape on the back (sticky paper technique on the back), the impression of 3 cm long duct tape on the abdomen (sticky paper technique on the abdomen) and by pulling hair from head, back and abdomen and combined (fur pluck technique). The samples were always taken in the same order; sticking on the back, sticking on the abdomen and pulling. Control for the presence of ectoparasites was performed one day before starting the treatment and monitored during the therapy before every administration of ivermectin. Based on the results of evaluating the sampling techniques, during and after treatment, the samples of hair were taken only with the fur pluck technique.

Evaluation of different sampling techniques

The fur pluck technique was used as the gold standard in defining the sensitivity of both sticky paper techniques for the detection of *Myocoptes musculinus*. The same procedure was used to evaluate the diagnostic potential for detecting *Myobia musculi* by all three techniques.

Data were analysed using Excel 2002 software. Differences among groups were analysed using Chi-squared / Fisher exact test with IBM SPSS Statistics program, version 20.Values of $P<0.05$ were considered to be significant.

Parasitological examination

Ectoparasites were examined under the stereomicroscope at 10× and 16× magnification. Samples obtained by pulling hair (fur pluck technique) or with impression of 3 cm long duct tape (sticky paper technique) were examined. Details of mites or their eggs were observed under a microscope at 40× and 100× magnification. Samples were previously soaked with 10 % KOH. Monitoring the presence of ectoparasites (adults, nymphs, larvae, and eggs) before, during and after the treatment was performed.

Results

Evaluation and the usefulness of different sampling techniques

Before treatment, all 59 (100 %) examined mice were confirmed to be positive for *Myocoptes musculinus* via the fur pluck technique. When we used sticky paper technique on the back and abdomen, 55 (93.2 %) and 54 (91.5 %) mice, respectively, were confirmed to be positive for *Myocoptes musculinus* (Table 1). The sensitivity of the sticky paper technique on the back and abdomen for *Myocoptes musculinus* was 91.5 and 93.2 %, respectively.

Before treatment, 37 (62.7 %) out of 59 examined mice were confirmed to be positive for *Myobia musculi* via the fur pluck technique. *Myobia musculi* was found on the backs of five mice (8.5 %) and on the abdomens of four mice (6.8 %) by using the sticky paper technique (Table 1). The results reveal the low sensitivity of the sticky paper technique (10.8 and 13.5 %, respectively) for *Myobia musculi.*

Eggs of *Myocoptes musculinus* were confirmed in all animals via the fur pluck technique before treatment, while it was determined in 89.8 % of the mice using sticky paper techniques. A total of 28.8 % of adults, nymphs, larvae and eggs were detected with the fur pluck technique and only 3.4 % to 8.5 % with sticky paper techniques. *Eggs* of *Myobia* musculi were confirmed in 61.1 % of animals via the fur pluck technique before treatment. Smaller percentages (from 6.8 % to 8.5 %) were determined via sticky paper techniques. Adults and their development forms were confirmed only via the fur pluck technique in 13.6 % (Table 2).

The effectiveness of therapy with ivermectin

Treatment of 59 mice was performed individually. Before, during and after the treatment, adults (N=39) and young mice (N=20) were separately monitored. Ivermectin therapy was monitored via parasitological examination, using the fur pluck technique. Table 3 shows the number of infested mice considering the age of mice.

The effectiveness of therapy with ivermectin for *Myocoptes musculinus* is statistically verifiable. A significant difference ($P<0.05$) in the proportion of infected mice after the second (75.4 %) and after the third (78.9 %) week of therapy was found in comparison with the number of infested mice with *Myocoptes musculinus* before treatment (100 %). The effectiveness of therapy against *Myobia musculi* was better than against *Myocoptes musculinus*; after the second and third weeks, only 24.3 and 35.1 % of mice, respectively, were still infected with *Myobia musculi*. The effectiveness of therapy with ivermectin is not evident after the first treatment for *Myocoptes musculinus* ($P=0.496$), nor is it for *Myobia musculi* ($P=0.396$). A significant decrease in the number of infected mice with *Myocoptes musculinus* is observed between the first and second (from 57 to 43) and between the first and third treatments (from 57 to 45) ($P<0.001$). The proportion of mice infected with *Myocoptes musculinus* and *Myobia musculi* did not differ from the second to the third week of therapy ($P=0.414$ and $P=0.153$, respectively).

All adult and young mice were infested with *Myocoptes musculinus* before the treatment, while a significantly lower proportion ($P<0.001$) of young mice (30.0 %) than adult mice (79.5 %) were infected with *Myobia musculi*. No difference in the proportion of infected mice with *Myocoptes musculinus* between adult and young mice was observed after the first treatment, but after the second and third treatments the proportion of infected mice was lower in young (31.6 and 36.8 %, respectively) than in adult mice (97.3 and 100.0 %, respectively). We found that even after the third treatment, all examined adult animals were still infested (mainly drained and damaged eggs were present, which cannot be seen from Table 3). Only eggs of *Myocoptes musculinus* were found in 36.8 % (7/19) of young mice. Treatment against *Myobia musculi* was more efficient in young than in adult mice ($P<0.001$); among adult mice, after the second and third weeks of therapy, 36.8 % and

Table 1: The number and percentage of positive samples in relation to sampling techniques

Sampling technique	No. of positive (percentage)*	
	Myocoptes musculinus	*Myobia musculi*
Fur pluck technique	59 (100.00)**	37 (100.00)**
Sticky paper technique on the back	55 (93.22)	5 (13.51)
Sticky paper technique on the abdomen	54 (91.53)	4 (10.81)

* No. (animals) = 59; samples were taken before the treatment.
** Fur pluck technique was established as gold standard.

Table 2: The number and percentage of positive samples in relation to parasite development forms and sampling techniques

Sampling technique	No. of positive (percentage)*							
	Myocoptes musculinus				*Myobia musculi*			
	All forms	Eggs	Adults, nymphs and larvas	N-infected (%)**	All forms	Eggs	Adults, nymphs and larvas	N-infected (%)**
Fur pluck technique	17 (28.8)	42 (71.2)	0 (0.0)	59 (100.0)	7 (11.9)	29 (49.2)	1 (1.7)	37 (62.7)
Sticky paper technique on the back	3 (5.1)	50 (84.7)	2 (3.4)	55 (93.2)	0 (0.0)	5 (8.5)	0 (0.0)	5 (8.5)
Sticky paper technique on the abdomen	1 (1.7)	52 (88.1)	1 (1.7)	54 (91.5)	0 (0.0)	4 (6.8)	0 (0.0)	4 (6.8)

* No. (animals) = 59; samples were taken before the treatment.
** Present both species of mites or singe infestation with Myocoptes musculinus.

Table 3: The number and percentage of positive samples before and after the treatment in all, adult and young mice

Time of collection		No. of positive (percentage)			
		Mixed infestation – *Myocoptes musculinus* and *Myobia musculi*	Single infection – *Myocoptes musculinus*	N (%) – infected*	N –all
All mice	Before treatment	37 (62.7)	22 (37.3)	59 (100.0)	59
	1st week	34 (58.6)	23 (39.7)	57 (98.3)	58**
	2nd week	14 (24.3)	29 (50.9)	43 (75.4)	57***
	3th week	20 (35.1)	25 (43.8)	45 (78.9)	57
Adult mice	Before treatment	31 (79.5)	8 (20.5)	39 (100.0)	39
	1st week	30 (76.9)	9 (23.1)	39 (100.00)	39
	2nd week	14 (36.8)	23 (60.5)	37 (97.3)	38**
	3th week	20 (52.6)	18 (47.4)	38 (100.0)	38
Young mice	Before treatment	6 (30.0)	14 (70.0)	20 (100.0)	20
	1st week	4 (21.0)	14 (73.7)	18 (94.7)	19***
	2nd week	0 (0.0)	6 (31.6)	6 (31.6)	19
	3th week	0 (0.0)	7 (36.8)	7 (36.8)	19

* Present both species of mites or singe infestation with Myocoptes musculinus.
** One adult mouse died
*** One young mouse died

52.6 %, respectively, were still infected, whereas no infected animals were observed in the group of young animals.

The number of mice infested with adult parasites and their development forms (including eggs) is also shown (Table 4). Using the fur pluck technique before the treatment, the eggs of *Myocoptes musculinus* were found on all 59 (100.0 %) mice: in 71.2 % of mice, only eggs were found; in 28.8% of mice eggs, adults, nymphs and larvae were observed. Among 37 mice (62.7 %) infested with *Myobia musculi*, 78.4 % (29/37) had only eggs, 2.7 % (1/37) had only adults, nymphs and larvae, and 18.9 % (7/37) mice had all forms. After the third treatment, 45 (78.9 %) mice were infested for *Myocoptes musculinus*. Among them, 82.2 % (37/45) had only eggs, 2.2 % (1/45) had only adults, nymphs and larvae, and 15.6 % (7/45) had all forms. For *Myobia musculi*, 20 (35.1 %) mice were positive and in all (100.0 %) only eggs were found. Almost all eggs of *Myocoptes musculinus* and *Myobia musculi* were drained and damaged after second treatment.

Discussion

Evaluation and the usefulness of different sampling techniques

Multiple diagnostic modalities exist. Optimal testing methods, target colony populations, or sampling sites are recommended (3). Fur pluck and sticky paper techniques for the diagnosis of *Myocoptes musculinus* and *Myobia musculi* in naturally infested mice were diagnostically evaluated (Table 1). Results before treatment show lower sensitivities of sticky paper techniques on the back and abdomen for *Myocoptes musculinus* (93.22 and 91.53 %, respectively) and *Myobia musculi* (13.51 % and 10.81 %, respectively) in comparison with the fur pluck technique. For this reason, the fur pluck technique was chosen as a gold standard for diagnosis during the treatment.

Using the fur pluck technique, we confirmed the presence of eggs of *Myocoptes musculinus* in all animals and in 89.8% of them by using both sticky paper techniques. With the use of sticky paper techniques, many false negative results for detecting adults and their development forms of *Myocoptes musculinus* were found. Only 8.5 and 3.4 % of animals were found to be positive for adults and their development forms by using the sticky paper technique on the back and abdomen, respectively, in comparison to the 28.8 % of those forms found with fur pluck technique.

From all mice, 62.7 % were positive for *Myobia musculi* according to the results from the fur pluck technique; eggs were found in 61.1 %, whereas adults and their development forms in 13.6 %. Only 8.5 and 6.8 % of mice were positive (only eggs were found in both cases) according to the results from the sticky paper techniques on the back and abdomen, respectively, showing a high level of false negative results when using sticky paper techniques for diagnosing infection with *Myobia musculi*.

We have established that sticky paper techniques are more suitable for sampling *Myocoptes musculinus* then *Myobia musculi*. Sticky paper techniques do not capture all *Myobia musculi* parasites, because of the way mites places eggs on hair. We have observed that *Myobia musculi* place eggs next to the skin, on the bases of two or more hairs together; consequently, the sampling is not always satisfying.

Hairs start to intertwine with each other in dense aggregations. Eggs are larger and heavier, lined along the base of the coat, close to the skin. They are lined along the thinner hairs or mostly on two thinner hairs, so it is more difficult to pick them up with glue. This is the main reason for the small percentage of positive mice for *Myobia musculi* confirmed by sticky paper techniques.

Interestingly, Metcalf Pate et al. (2011) found that sticky paper techniques were more likely to detect fur mites than the fur pluck technique was. They found that the surface temperature of the murine neck surface was warmer than the rump was and thus may represent a unique microenvironment for the development of fur mite eggs. Authors recommend that group-housed adult or preweanling mi ce should be selected for *Myocoptes musculinus* evaluation and that the abdomen should be sampled (3).

The effectiveness of therapy with ivermectin

The treatment of common infestations with mites in conventional colonies of laboratory mice is frequently problematic. The choice of acaricide, the method of application, and the treatment intervals are important in the control of these ectoparasites. Acaricides generally have no effect on acarine eggs. For this reason, re-treatment

after 8–16 days is recommended (4, 9, 14). Wing et al. (1985) concluded that reinfestation must occur if the efficiency of ivermectin ceased before all eggs had hatched (5). In the current study, mice were treated three times at intervals of seven days. Reported ivermectin doses for mice vary from 20 to 400 µg/kg and appear to have been chosen arbitrarily (6). We used ivermectin at a dose 200 µg/kg on BW, similar to the study of Arbona et al. (6, 7).

Ivermectin is one of the most common parasiticides used to treat fur mites (7, 9). It can be administered topically on mice, spraying individual cages of mice, orally in food and water, or by parenteral application administered subcutaneously (4, 6, 7, 11). Davis et al. (15) recorded that ivermectin did not affect the general health, body weight, motor coordination, or spatial learning in several inbred strains of mice. However, it induced a small but significant effect on some sensitive behaviour. At the end of the treatment, we confirmed the presence of *Myobia musculi* eggs in the faeces of one mouse. This proves that mice lick the fur and eat hair, with eggs glued on them and, indirectly, also the drug, which is topically applied on the fur. However, in our study, changes in behaviour were identified neither in adults nor in young mice, and no signs of health problems were observed. The only negative property of ivermectin was a relatively long withdrawal period. It cannot be used on mice intended for the feeding of reptiles for 30 days.

Before treatment, all adult and young mice were infected with *Myocoptes musculinus*, whereas infection with *Myobia musculi* was found in 79.5 % of adults and only in 30.0 % of young mice. Treatment with ivermectin was more effective in young mice. At the end of the treatment period, all examined adult animals were still infected, although mainly drained and damaged eggs were present, whereas only eggs of *Myocoptes musculinus* were found in 36.8 % of young mice. Moreover, with regards to *Myobia musculi*, no infected animal was observed in a group of young animals after the end of the treatment, but 52.6 % of adult mice were still infected (Table 3).

The percentage of infected mice with adults, nymphs and larvae of *Myocoptes musculinus* significantly decreased from 28.8 % before the treatment to 14.0 % after the third treatment (P=0.043) and the percentage of mice in which only eggs were found decreased from 71.2 % before the treatment to 64.9 % after the third treatment without significant difference (P=0.300). Based on the results obtained for Myobia musculi, we have concluded that ivermectin is effective in the used dosage for adult forms of Myobia musculi. A week after the third treatment was completed, only eggs were found (Table 4).

Before and after the treatment, the largest number of mice was infested with eggs. However, after the second application of ivermectin almost all had damaged outer membrane walls. This was regularly observed after the third treatment with ivermectin. In our study, whether eggs were able to survive was not checked. Arbona et al. (2010) assumed that mite eggs and casings can persist for months in mice presumptively treated successfully for fur mites, because eggs are attached permanently to hair shafts and potentially could remain on the animal until the affected hair is shed. They stated that it is extremely difficult to determine whether these mite eggs were viable and whether they had been laid before or after the treatment. Some nonviable eggs could remain until the entire hair coat is shed (16). The presence of mite parts, nonviable eggs, or egg casings, which can remain attached to hair for several months after successful treatment, make it difficult to verify treatment success (7). Techniques for the determination of egg viability were described by Burdett et al. (4, 11). For this reason, it is essential to include confirmation of the vitality of eggs, development forms and adults in routine parasitological diagnostic procedures. We assume that at the end of the third treatment eight mice, in which all forms (7) or adults, nymphs and larvae (1) were, found were still able to infect others with *Myocoptes musculinus*. Therefore, we assume that testing of 59 mice revealed that the efficiency of treatment using this dose of ivermectin for *Myocoptes musculinus* was 86 % effective after three repetitions of treatment.

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ZDRAVLJENJE AKARIAZE Z IVERMEKTINOM IN OCENA RAZLIČNIH METOD VZORČENJA PRI MIŠIH

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Povzetek: Vzrejnim mišim, pozitivnim na *Myocoptes musculinus* in *Myobia musculi*, smo akariazo zdravili z ivermektinom v odmerku 200 µg/kg telesne mase (6µl aktivne snovi). Ivermektin smo aplicirali lokalno na kožo v področje zatilja. Aplikacijo smo ponovili trikrat v enotedenskih intervalih. V skupinah odraslih in mladih miši smo pred zdravljenjem, med njim in po njem ugotavljali pristonost različnih razvojnih oblik pršic (odraslih, nimf, ličink in jajčec). Pred zdravljenjem sta bili ovrednoteni dve metodi za zaznavanje različnih oblik pršic: metoda puljenja dlake in metoda lepljenja dlake (vzorčili smo na dveh različnih mestih; na hrbtu in trebuhu). Tehnika puljenja dlake je bila uporabljena kot zlati standard. Občutljivost metode lepljenja dlake je bila 91,5 - 3,2% za *Myocoptes musculinus* in 10,8 - 13,5% za *Myobia musculi*. Ugotovili smo, da *Myobia musculi* odlega jajčeca tik ob koži, na bazi dveh ali več dlak skupaj, zato vzorčenje ni vedno zadovoljivo. Učinkovitost zdravljenja z ivermektinom na *Myocoptes musculinus* in *Myobia musculi* je bila potrjena po drugem zdravljenju. Pred zdravljenjem, med njim in po njem smo opazili značilno višji odstotek pozitivnih miši pri starih živalih v primerjavi z mladimi. Po tretjem zdravljenju smo še vedno ugotavljali tako odrasle kot tudi razvojne oblike (jajčeca, ličinke in nimfe) *Myocoptes musculinus*. Skoraj vsa jajčeca *Myocoptes musculinus* in *Myobia musculi* so bila po tretjem zdravljenju izsušena in poškodovana.

Ključne besede: veterinarska medicina; ektoparaziti; *Myocoptes musculinus*; *Myobia musculi*; zdravljenje

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Case Report

Enterococcus cecorum INFECTION IN TWO CRITICALLY ILL CHILDREN AND IN TWO ADULT SEPTIC PATIENTS

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Summary: *Enterococcus cecorum* is mostly found as normal gut flora in farm animals, especially pigs and poultry. However, sometimes it can cause extended infections and disease in those animals, as was recently reported in Canada where it caused arthritis and osteomyelitis in chickens. Until now, only a few reports have been published on *Enterococcus cecorum* as a potential pathogen in humans. We have reported 4 cases of infection with this rare human pathogen.

The organism was proven in 2 blood samples from adult patients with sepsis and in 2 cerebrospinal fluid (CSF) samples taken from children with external ventricular drainage (EVD) and diagnosed ventriculitis. In two cases (one child and one adult), other bacterial pathogens were also detected. The organism could not be cultivated and could only be identified with analysis of 16S rRNA gene PCR. The following molecular biomarkers were used to confirm the infection, and exclude sample contamination: white blood cell count, neutrophils, C-reactive protein (CRP), procalcitonin (PCT) and presepsin (sCD14-ST).

Enterococcus cecorum was identified as a pathogen with 16S rRNA gene PCR and could have caused the infection in all patients. We also suspected the first possible human-to-human transmission of bacteria from a mother to a newborn child.

Key words: *Enterococcus cecorum*; broad-range 16S rRNA gene PCR; infection in children and adults; human-to-human transmission; presepsin sCD14-ST

Introduction

Enterococcus cecorum has previously been classified as *Streptococcus cecorum* and was renamed *Enterococcus cecorum* after sequencing of its 16S rRNA gene. Phenotypically it is often mistakenly described as *Streptococcus* group D. In addition to conventional cultivating tests, it is possible to prove infection with proteins, analysis of fatty acids from culture or 16S rRNA gene sequencing [1]. *Enterococcus cecorum* is a normal inhabitant of the intestines of birds and other vertebrates. It recently emerged in Canada and some other countries as an important cause of arthritis and osteomyelitis in chickens [2], isolated from the intestines of poultry, pigs, cattle, cats and dogs as part of normal gut flora [3, 4]. Human infections were diagnosed in rare cases. The first human case was reported in 1997 when *E. cecorum* was isolated from the blood of a patient with severe septicemia by conventional biochemical tests. Its identity as *E. cecorum* was confirmed by SDS-PAGE analysis of whole cell proteins [1]. Furthermore, bacteria were isolated as an etiologic agent of infection from a patient on continuous ambulatory peritoneal dialysis with episodes of peritonitis [5], later from a 60-year-

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old man with liver cirrhosis and hepatocellular carcinoma who then developed peritonitis [6], from a man with empyema thoracis successfully treated with cefotaxime [7] and from a 44-year-old man with infectious aortic valve endocarditis [8]. In the last case, *E. cecorum* was mistakenly identified as *Streptococcus salivarius* from blood culture and was later confirmed as *E. cecorum* from a valve tissue sample with 16S rRNA gene PCR.

In this article, we described 4 cases of *E. cecorum* as a cause of (co)infection. We discovered *E. cecorum* as a potential cause of sepsis in 2 adult immunocompromised patients and in 2 children with ventriculitis, who had increased intracranial pressure (ICP) and needed external ventricular drainage (EVD). In all the cases, we used 16S rRNA gene sequence analysis to prove the presence of *E. cecorum* as a pathogen.

Case reports

A 63-year-old Caucasian woman on hemodialysis due to kidney failure and type II diabetes was brought to a general hospital with a short history of increasing breath shortness associated with fever and rigors in May 2011. The woman was accepted to the general hospital's intensive care unit with suspected sepsis. Four years before admittance, she had a massive ischemic stroke and was admitted to a care unit for the elderly and disabled. No history of animal contact and travelling abroad was reported. At presentation, the patient was pyrexial (39.1 °C), hypotensive, with signs of septic shock, her heart rate was > 150 beats per minute and her respiratory rate was > 20 breaths per minute. She had ulcers on her left thigh and sacral region. The patient was subsequently commenced on empirical cefazolin and then switched after 5 days of hospitalization to piperacillin/tazobactam. After 20 days of hospitalization at the intensive care unit, she got better, her vital signs improved, the values of biomarkers lowered and she was discharged.

A 66-year-old male patient was admitted to the intensive care unit of a general hospital because of acute respiratory distress syndrome (ARDS) and septic shock. He has been treated in the rheumatology ward for severe knee arthritis and leukemia since 2003. It transformed to myelodysplastic syndrome in 2010.

A 9-month-old male child with raised ICP due to an operation of a cystic lesion in the brains and installed EVD was treated at an intensive care unit of a tertiary clinic. The child had 3 episodes of fever with epileptic attacks and vomiting afterwards, which developed due to bacterial ventriculitis/meningitis. The last episode was 6 weeks after the operation. The child was still on meropenem and the treatment was switched to gentamycin and vancomycin after the pathogens were identified.

A newborn male with raised ICP due to subdural hemorrhage and installed EVD was treated in June 2012. The mother was healthy and the baby was born on term with an Apgar score of 9/10. The baby was transported from the general hospital to a tertiary intensive care unit because of apneic attacks 3 days after birth. The baby was still in the hospital with the mother and they were both transferred to another hospital where the facilities to treat such newborns were possible. Subdural hematoma was discovered and it was decided that the baby needed EVD due to raised ICP. The catheter for monitoring cranial pressure was removed after 5 days and on the 8th day pus was discovered around the drainage device. The biomarkers of infection were also taken, but the analysis of CSF was inconclusive.

Methods and results

All the patients were admitted to intensive care units between May 2011 and June 2012. The blood to set the values of infection biomarkers and for blood cultures was collected. The observed markers of infection were: white blood cell count, percentage of neutrophils, C-reactive protein (CRP, Siemens, Germany), procalcitonin (PCT, Brahms, Germany) and sCD14-ST (presepsin) in CSF of two children (Mitsubishi Chemical Europe, Germany) (Table 1). Respiratory tract samples and urine were taken for cultures. The blood cultures were negative in 3 cases. We isolated *S. pneumonia* in the case of a 66-year-old man (Table 1). In the case of a 9-month-old child with raised ICP, *Serratia marcescens* from CSF was isolated. Because of clinical suspicion of bacterial infection, broad-range 16S rRNA gene PCR to prove the causing pathogen was performed in duplicates. Bacterial DNA was extracted from a whole blood sample or CSF using SepsiTest™ (Molzym, Germany). Isolation and PCR amplification were performed following the

manufacturer's instructions. Sequences of the PCR product were compared with GenBank 16S rRNA gene sequences available at the National Centre for Biotechnology Information and Ribosomal Database Project using BLAST algorithm and with SepsiBlast program (Molzym, Germany) [9, 10]. In all 4 cases, the sequence score for *E. cecorum* was 100% and the sequence coverage with the available ones was not less than 99%.

Discussion

Few cases of infection with *E. cecorum* have recently been reported in English literature and all patients had underlying diseases [8]. We reported the first 2 cases of presence of *E. cecorum* in children and 2 cases of possible sepsis in adult immunocompromised patients, like in the study from Tan et al. [11]. The patients' data is presented in Table 1. In our cases, conventional methods could not identify *E. cecorum* from stool samples because standard routine microbiological methods are not able to differentiate *E. cecorum* from other enterococcal species [11]. All our patients were on broad-spectrum antibiotics for 24 hours before the samples for cultures were taken, so the therapy could explain why we could not identify the cause of infection with conventional methods.

In 2 patients, bacteria other than *E. cecorum* were also identified with broad-range 16S rRNA PCR. In one child, *S. marcenscens* was cultured from CSF, which definitely contributed to the severity of the child's infection, and *E. cecorum* was the only (co)pathogen which might have contributed to the severity of infection. Furthermore, in the case of a septic adult, *S. pneumoniae* was identified with 16S rRNA gene PCR as the cause of infection and most probably resulted in disease severity and pneumonia. The biomarkers which were used to prove bacterial infection were elevated and excluded other potential causes of infection like viruses and fungi or sample contamination by potential normal bacterial flora from the skin. They were above the limit of positivity for bacterial infection in all cases (Table 1). However, the use of conventional infection biomarkers did not help in the case of 2 children. We found normal levels of leukocytes, CRP and PCT in whole blood in a newborn and in a 9-month-old child with ventriculitis [12]. In a recent article, authors described the use of sCD14-ST to identify septic patients. The concentration of sCD14-ST was significantly higher in the sepsis group than in the healthy group [13]. We used the same analogy for CSF and assumed that a value of sCD14-ST above 1000 pg/mL was a good marker to confirm ventriculitis. In the case of the newborn, all conventional biomarkers failed to prove the infection. Only the use of sCD14-ST revealed that the newborn could have bacterial ventriculitis. In the case of the 9-month-old child with bacterial ventriculitis/meningitis, the cells and proteins in CSF were increased, the glucose levels were lower than normal and sCD14-ST was also raised. We have not been able to measure the sCD14-ST in blood from children since we could not obtain the samples.

Conclusion

It was recently reported that poultry in Canada and Europe got infected with *E. cecorum*. It was never proven that animal-human transfer had happened [2, 4, 14]. All our patients came from rural areas where small family farms raise a great variety of farm animals including pigs and poultry. We assumed that farm animals were a possible source of *E. cecorum* but could not prove our thesis, since prevalence in humans is only 0.1% [11]. We also assumed that people could also carry *E. cecorum*, as is the case in *E. faecalis*, in their gut or on their skin. We had a case of a newborn with ventriculitis, possibly caused by *E. cecorum*, the only organism proven. We assumed that only his mother, who lived in a rural hilly area where they raise farm animals and come into indirect contact with them, could be the source of bacteria. We lacked strong epidemiological data that human-to-human transmission had happened. The newborn was transferred from one hospital to another and had never come in contact with animals during the stays. In our opinion, human-to-human transmission was thus the only explanation of how *E. cecorum* could come into CSF through EVD and cause bacterial ventriculitis. In the case of isolation of *E. cecorum* in humans from sterile places, contamination of samples from the environment is possible. In such cases, we recommend the use of bacterial infection biomarkers such as sCD14-ST that show an activating immune system response to distinguish between contaminations and

Table 1: The patients' data

Gender	Age	Contact with animals	Admittance diagnosis	Co-morbidity	Cultures	16S rRNA gene analysis from whole blood or CSF	Sequence score/ coverage for E. cecorum	Biomarkers in blood leuco/neutro/ CRP/PCT	Biomarkers in CSF (leuco, proteins, glucose, sCD14-ST)	Outcome
Female	63 years	Lived in a rural area on a farm. Lived in a care unit for the elderly and disabled during the last years of her life.	Sepsis	End kidney failure on hemodialysis	Blood cultures negative	*Enterococcus cecorum* from blood	100% 302bp of 302 (100%)	36.2 × 10^9/L 32.7 × 10^9/L 153 mg/L 0.98 µg/L Raised		Died of uncontrolled diabetes and kidney failure two months after discharge
Male	66 years	Lives in a village with many farms, has a flock of chickens, possible animal-to-human transfer.	Septic shock	Leukemia with transformation to myelodysplastic syndrome	Blood cultures negative	*S. pneumoniea* and *Enterococcus cecorum* from blood	100% 410bp of 412 (99%)	8.6 × 10^9/L 8.2 × 10^9/L 372 mg/L 1.82 µg/L Raised		Good and discharged from the hospital
Male	9 months	Lives in a rural area, contact with farm animals possible.	Bacterial ventriculitis/ meningitis	Subdural hematoma with raised ICP and EVD	*Serratia marcenscens* from CSF	*Enterococcus ceco rum* and *Serratia marcenscens* from CSF	100% 412bp of 412 (100%)	10× 10^9/L 3,8× 10^9/L 14mg/L 0,04 µg/L Normal	Typical of bacterial meningitis, 1631 pg/mL	Good and discharged from the hospital
New-born male	3 days	Mother from a village with many farms. Newborn transferred directly from a regional general hospital to a university clinical center.	Bacterial ventriculitis	Cystic lesion in the brain and EVD	Negative CSF	*Enterococcus cecorum* from CSF	100% 413bp of 417bp (99%)	5,3× 10^9/L 2,5× 10^9/L 5mg/L 0,9 µg/L Normal	Normal, 1381 pg/mL	Good and discharged from the hospital

Leuco-leucocytes, neutro-neutrophils, CRP C–reactive protein (normal value less than 5mg/L), PCT-procalcitonin (normal values less than 0,5 µg/L), CSF-cerebrospinal fluid, presepsin sCD14-ST (blood value above 1000pg/mL is typical of septic shock)

true infections, especially if the highly sensitive molecular method of 16S rRNA broad-range PCR is used.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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OKUŽBA Z *Enterococcus cecorum* PRI DVEH KRITIČNO BOLNIH OTROCIH IN DVEH ODRASLIH PACIENTIH S SEPTIKEMIJO

David Štubljar, Miha Skvarč

Povzetek: *Enterococcus cecorum* večinoma najdemo kot del normalne črevesne flore domačih živali, zlasti prašičev in perutnine. Kljub temu pa mikroorganizem lahko povzroči okužbo in razvoj bolezni kot pomemben vzrok artritisa in osteomielitisa pri piščancih, kot je bil zabeležen primer v Kanadi. Do sedaj je bilo objavljenih le nekaj poročil o *Enterococcus cecorum* kot potencialnem patogenu človeka. Tukaj poročamo o štirih primerih redke okužbe s človeškim patogenom *Enterococcus cecorum*. Organizem smo dokazali v dveh krvnih vzorcih odraslih bolnikov s sepso in v dveh vzorcih cefalospinalne tekočine, odvzete pri otrocih z zunanjo ventrikularno drenažo in diagnosticiranim ventrikulitisom. Pri enem otroku in enem odraslem bolniku so bili prisotni tudi drugi bakterijski patogeni. Organizem smo lahko določili le z analizo gena 16S rRNK. Za potrditev okužbe smo določili molekularne biološke označevalce, kot so C-reaktivni protein (CRP), prokalcitonin (PCT) in presepsin (sCD14-ST), in s tem izključili možnost kontaminacije vzorca. Domnevamo, da je prišlo do mogočega prenosa bakterije od matere na novorojenčka, kar bi predstavljalo prvo poročilo o prenosu s človeka na človeka.

Ključne besede: *Enterococcus cecorum*; širokospektralni PCR; gena 16S rRNA; okužba pri otrocih in odraslih; prenos okužbe s človeka na človeka, presepsin sCD14-ST

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