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IMMUNOCHROMATOGRAPHIC DETECTION OF BOVINE ROTAVIRUS USING EGG YOLK ANTIBODIES*

IMUNOCHROMATOGRAFICKÁ DETEKCE BOVINNÍHO ROTAVIRU S VYUŽITÍM PROTILÁTEK VAJEČNÉHO ŽLOUTKU

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ABSTRACT: An immunochromatographic test (ICT) for the detection of bovine rotavirus in fecal samples using the specific anti-rotavirus colloid gold-bound immunoglobulin isolated from egg yolks (IgY) of immunized hens has been developed. The yield of IgY was 100 to 150 mg per yolk and ELISA antibody titers ranged between 50 000 and 100 000. ICT was used for 130 fecal samples collected from calves aged from 1 to 50 days. A comparison with ELISA showed 90.7% agreement. The values of specificity and sensitivity were 97.3% and 82.1%, respectively. In an analogous comparison with Dot-ELISA, the agreement, specificity and sensitivity values obtained for ICT were 96.9%, 96.4%, and 97.8%, respectively. Detection of rotavirus double-stranded RNA by agar-gel electrophoresis resulted in 90.3% agreement with ICT.

immunochromatographic test (ICT); ELISA; Dot-ELISA; egg IgY; bovine rotavirus

ABSTRAKT: Pro diagnostiku rotaviru ve fécés telat byla použita imunochromatografická metoda (ICT) využívající specifický antirotavirový imunoglobulin izolovaný z vaječných žloutků (IgY) imunizovaných nosnic. Výtěžnost IgY dosahovala hodnot až 150 mg na jeden žloutek s titrem antirotavirových protilátek až 100 000. Získaný IgY byl vázán na koloidní zlato. Pomocí ICT bylo vyšetřeno 130 vzorků fécés telat. Porovnáním výsledků s metodou ELISA bylo dosaženo celkové shody 90,7 %, byla vypočtena specifita 97,3 % a citlivost 82,1 %. Srovnáním s Dot-ELISA byla shoda 96,9 %, specifita 96,4 % a citlivost 97,8 %. Nižší záchyt pozitivity v ICT se týkal výhradně vzorků detekovaných v blízkosti cut-off linie v ELISA. Vybraný soubor 31 vzorků fécés byl vyšetřen elekroforézou v agarózovém gelu na přítomnost segmentů rotavirové RNA. Celkem 90,3 % výsledků bylo totožných jako v ICT.

imunochromatografická metoda (ICT); ELISA; Dot-ELISA; vaječný IgY; bovinní rotavirus

INTRODUCTION

The tendency in diagnostics of infectious diseases is to develop methods with a high sensitivity and specificity. However, advanced methods are often time-consuming and/or require an equipment that is not available to all diagnostic laboratories. Therefore, the research concentrates on simple and rapid procedures with adequate sensitivity and specificity. Examples thereof are Dot-ELISA (Chauhan, 1992) and immunochromatography (Gupta et al., 1992). The principle of the latter technique consists in the conjugation of specific antibodies with color microspheres (color latex particles or colloid gold), formation of a corpuscular complex with the antigen tested, and its penetration by capillary lift through a membrane carrying irreversibly bound second antibody complementary to the antigen to form a color line or spot (Gupta et al., 1992; Dar et al., 1994; Bhaskar et al., 1996; Garcia, 1996; Klingenberg and Esfandiari, 1996; Singh et al., 1997; Torlesse et al. 1997; Durrheim et al., 1998). The same principle allows the detection of specific antibodies or their isotypes to antigens of various infectious agents (Schrier et al., 1998; Vaughn et al., 1998). Like in other immunological methods, the sensitivity and specificity of the immunochromatographic test (ICT) depends on the characteristics of antibodies used in it. Polyclonal or monoclonal antibodies alone or in combinations are used for the conjugation with the microspheres and for the binding onto the membranes (Dar et al., 1994). Increased attention is currently paid to hen's eggs as an alternative source of specific antibodies because immunization of hens induces approximately equal concentrations of egg yolk and blood serum antibodies (Schmidt et al., 1989; Gassmann et al., 1990; McLaren et al., 1994). Egg yolk immunoglobulin (IgY) can be isolated by a simple and high-yield procedure (Jensenius et al., 1981; Hatta et al., 1990; Akita and Nakai, 1993). Due to the phylogenetic distance between birds and mammals, egg yolk antibodies show less non-spe-

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cific reactions in mammalian species and have been successfully used in various diagnostic tests (Schmidt et al., 1989; Hlinak et al., 1996).

The aim of our experiment was to combine the merits of both IgY antibodies and the one-step immunochromatographic technique to develop a rapid and simple diagnostic method for the detection of group A rotavirus infections in calves.

MATERIAL AND METHODS

Rotavirus

Group A rotavirus CAPM V-279 (strain Lincoln) was propagated in the MA-104 cell line maintained by passaging in the Eagle MEM growth medium supplemented with 5 to 10% bovine fetal serum as described by Dar et al. (1994). The infected cultures were further maintained in the non-supplemented Eagle MEM maintenance medium. The cultures were frozen/thawed once after 24 to 48 h of incubation and cell debris was removed by centrifugation at 7 000 r.p.m., rotor JS 7.5, for 20 min using the J2-21 centrifuge (Beckman Instruments, Palo Alto, USA).

A part (500 ml) of the supernatant containing the virus particles was used as the positive control antigen in ELISA. The negative control antigen was prepared from mock-infected cell cultures using the same procedure.

Another part (500 ml) of the supernatant was centrifuged at 25 000 r.p.m. and 10 °C for 1.5 h using the rotor Sw 28 (Czerny and Eichhorn, 1989). The sediment was resuspended in PBS, pH 7.2, transferred onto a layer of 30% saccharose and centrifuged at 35 000 r.p.m. and 10 °C for 2 h using the rotor Sw 55 Ti (centrifuge L8-80M, Beckman Instruments, Palo Alto, USA). The purified virus was used for the immunization of rabbits and hens.

Antibodies to rotavirus A (strain Lincoln)

Two parts of the purified virus suspension were mixed with one part of the complete (for 1st inoculation) and incomplete (for subsequent inoculations) Freund's adjuvant.

Californian rabbits aged 3 months were immunized subcutaneously three times at 3-week intervals with a dose of 0.5 ml containing 50 µg of viral proteins distributed in four sites, and destroyed and bled one week after the last treatment. Immunoglobulin with antibody activity was obtained using the standard separation procedure with 33%-saturated ammonium sulfate solution.

ISA Brown hens aged 20 weeks were immunized intramuscularly with the same dose as above five times at two-week intervals. IgY antibodies to rotavirus were isolated from egg yolks laid from day 50 after the beginning of the inoculation as described by Jensenius et al. (1981). Briefly, this included the removal of lipoproteins with dextran sulfate and subsequent precipitation with 40% ammonium sulfate. ELISA was used to check the concentrations of the antibodies.

Fecal samples

A total of 130 fecal samples were collected in six cattle herds affected by neonatal enteritis. The fecal samples were suspended in a fivefold volume of PBS, pH 7.2, homogenized and centrifuged at 7 000 r.p.m. for 20 min in the centrifuge B4i (Jouan, France). The supernatants were stored at –20 °C until use.

Conjugation of anti-rotavirus IgY with colloid gold

Colloid gold was prepared from 0.02% gold chloride using sodium citrate as described by Gupta et al. (1992). Only the solutions with absorption maxima between 525 and 535 nm in spectrophotometrical measurements at 450 to 700 nm were eligible for conjugation.

The colloid gold solution pH was adjusted to 6.5 with 1% potassium carbonate and 1 ml was mixed with 5 µg/ml of anti-rotavirus IgY. After 5 to 10 min of incubation, the solution was completed with 6 µl of 20% BSA, transferred into a plastic centrifuge tube and centrifuged for 10 min at 6 000 r.p.m. in the centrifuge B4i. The supernatant was withdrawn, but 50 µl were left in the centrifuge tube and the sediment was resuspended.

Immunochromatography (ICT)

A nitrocellulose membrane (pore size 8 µm, Sartorius, USA), bound to a plastic support (0.5 x 5.0 cm), was used in the immunochromatographic test. Three µl of polyclonal rabbit anti-rotavirus antibody containing the 50 µg per ml 0.01 M PBS, pH 7.2, was applied to one end of the membrane (test zone) and the same volume of polyclonal antibody to IgY, containing 200 µg per ml 0.01 M PBS, pH 7.2, (control zone) was applied at a distance of 5 mm from the former. After drying, the surface of the membrane was blocked with 1% BSA in 0.01 M PBS, pH 7.2.

Twenty µl of IgY antibodies conjugated with the gold microparticles and mixed with 40 µl of PBS, pH 7.2, containing Triton X-100 and saccharose in an amount supporting the capillary lift, was applied into a flat-bottomed microtitre plate well along with the 40 µl of tested sample. After mixing, a strip of the membrane was placed into the well. Negative samples did not induce any color change in the test zone. The positive reaction resulted in a formation of color spot as a consequence of the reaction between immobilized anti-IgY antibody and gold-conjugated IgY. The results were

read 5 min after placing the membranes into the well containing the sample.

Dot-ELISA

The commercial diagnostic set Dot-ELISA Rota strip (Test-Line Diagnostics Ltd., Brno, Czech Republic) was used according to the manufacturer's instructions. Briefly, 20-min incubation of a test strip in the sample of fecal suspension was followed by triple washing and binding the rabbit anti-rotavirus conjugate to the formed immune complex. After another triple washing, the strip was immersed into a diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, USA) substrate + hydrogen peroxide solution for 2 to 5 min, washed three times again and dried. The reaction was read visually and the sample was scored as positive if an intensive reddish-brown spot, distinguishable from the light background, developed in the middle of the strip.

ELISA

The direct sandwich technique was used. Each binding step was followed by triple washing with PBS. Rabbit antibodies to rotavirus at a concentration of 15 μg/ml of 0.05M carbonate-bicarbonate buffer, pH 9.6, were bound to the surface of polystyrene 96-well microtitre plates (GAMA, České Budějovice, Czech Republic). The suspensions of fecal samples in PBS were tested in twofold dilutions starting with 1 : 2. Positive and negative controls were run in each plate. Horse radish peroxidase-labeled rabbit antibodies to rotavirus (Farr and Nakane, 1981) diluted 1 : 4 000 were pipetted into the wells as the conjugate. Tetramethyl benzidine (Sigma Chemical Co., St. Louis, USA) as the substrate solution and hydrogen peroxide were added and the resulting reaction was stopped with 1M sulfuric acid. The intensity of the resulting yellow color was measured spectrophotometrically at 450 nm and samples (dilutions) showing absorbances > 0.100 were scored as positive (Beards, 1984).

Detection of rotaviral RNA in agarose gel (EPA)

The method described by Pšikal et al. (1991) and Dvořák et al. (1993) was used. If present, segments of rotaviral ds RNA were detected with 1% ethidium bromide in ultraviolet light at 312 nm (Transilluminator UVP, Hoefer, Scientific Instruments, USA).

RESULTS

Rotavirus-specific IgY was separated from egg yolks of inoculated hens. It yielded 100 to 150 mg of rotavirus antibodies per yolk and ELISA titers ranged between 50 000 and 100 000. Regular checks have shown that the antibodies could be stored at –20 °C for at least two years without any significant loss of activity.

Comparisons were done by using a randomly selected subset of 31 samples (5 to 6 recently collected samples per herd) by electrophoresis of rotaviral ds RNA segments in agarose gel (EPA) and the results were compared with those of ICT, Dot-ELISA, and ELISA. The results of EPA agreed with those of ICT and Dot-ELISA in 90.3% and with those of ELISA in 87.1% (Tab. I). Samples with low concentrations of rotavirus accounted for the higher percentage of samples positive by ELISA only (Tab. II).

Results of comparative trials of ICT, ELISA and Dot-ELISA, done in the whole set of 130 samples, are presented in Tab. III. It can be seen that 2 and 3 of the 48 samples positive by ICT were negative by ELISA and Dot-ELISA, respectively. On the other hand, 10 and 1 of the 82 samples negative by ICT were positive by ELISA and Dot-ELISA, respectively. The overall agreement of the results was 90.7% for ICT vs. ELISA and 96.9% for ICT vs. Dot-ELISA. ELISA yielded a higher percentage of positive results. Compared with ELISA, the sensitivity and specificity values for ICT were 97.3% (72 of 74) and 82.1% (46 of 56), respectively. Compared with Dot-ELISA, the corresponding values were 96.4% (81 of 84) and 97.8% (45 of 46), respectively.

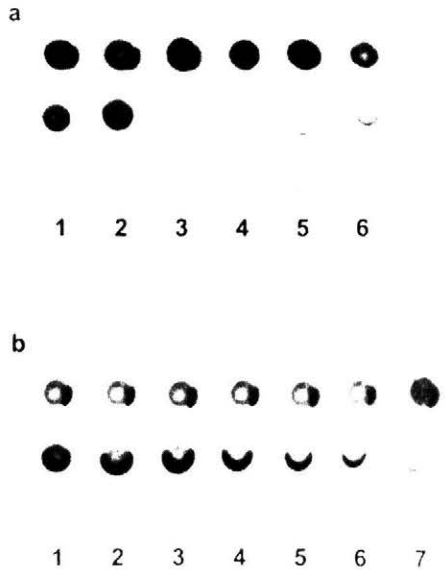
The distribution of contradictory results of ICT and ELISA is shown in Tab. II. It can be seen that such results were obtained only in samples with ELISA titers <16, i.e. those with a lower content of rotavirus. Full agreement (100%) was obtained in all the samples with ELISA titers ≥ 32.

I. Comparison of results of rotavirus detection by EPA with those of ICT, ELISA, and Dot-ELISA (selected samples, $n = 31$)

EPA		ICT		Dot-ELISA		ELISA	
+	–	+	–	+	–	+	–
12		12	0	12	0	12	0
	19	3	46	3	16	4	15
Total: 31		15	16	15	16	16	15

II. Comparison of results of rotavirus titration by ELISA with those of ICT and Dot-ELISA in 130 samples

ELISA		ICT		Dot-ELISA	
Titre	n	+	–	+	–
0	74	2	72	3	71
2	7	2	5	2	5
4	4	1	3	1	3
8	2	1	1	0	2
16	5	4	1	3	2
32	5	5	0	4	1
64	8	8	0	8	0
128	6	6	0	6	0
256	9	9	0	9	0
512	10	10	0	10	0
Total	130	48	82	46	84



1. Immunochromatographic detection of rotavirus; a: strongly positive (1–2), negative (3–4), and weakly positive (5–6) samples, b: titration of a rotavirus suspension containing 109 particles per 1 ml; dilution series 1 : 20 to 1 : 1 280 (1–7)

III. Comparison of results of rotavirus detection by ICT, ELISA, and Dot-ELISA (complete set of samples, n = 130)

ICT		ELISA		Dot-ELISA	
+	–	+	–	+	–
48		46	2	45	3
	82	10	72	1	81
Total: 130		56	74	46	84

The sensitivity of ICT was tested using twofold dilutions of a suspension containing ~10^9 rotavirus particles per 1 ml. The results were positive up to the dilution 1280, as can be seen in Fig. 1 showing also characteristic patterns yielded by strongly positive, weakly positive, and negative fecal samples.

DISCUSSION

The applicability of ICT for the detection of rotavirus antigens in fecal samples collected from calves aged from 1 to 50 days was tested. Unlike other methods based on the antibody-antigen reaction, ICT is the one-step procedure that does not require separation of free antibodies from the antibody-antigen complex and subsequent quantification steps associated with changes in optical density measured by spectrophotometry. The merit of ICT is its simplicity and speed since results can be obtained within 10 min after the application of the tested samples (Gupta et al., 1992; Klingenberg and Esfandiari, 1996). The limiting factors in the optimization of ICT is the choice of a membrane substrate in respect to pore size, antibody binding and humidifying capacity (Lagastelois, 1996).

A combination of monoclonal and polyclonal antibodies is often used in ICT (Dar et al., 1994; Klingenberg and Esfandiari, 1996; Torlesse et al., 1997). In our study we used the polyclonal IgY antibodies that might reduce the risk of non-specific reactions (Larson et al., 1993; Rieger et al., 1996). IgY were isolated from egg yolks with a yield comparable with the data published by Schade and Hlinak (1996) and Schwarzkopf and Thiele (1996).

ICT, ELISA and Dot-ELISA are procedures based on the visualization of the antibody-antigen reaction, while EPA detects the presence of viral ds RNA. This difference in the detection principle might be a factor responsible for some discrepancies between the EPA results and those of the other three methods. It should be stressed, however, that none of the samples positive by EPA was scored as negative by any of the remaining three methods.

The results of ICT and Dot-ELISA are read visually and scored on the "dot/no dot" color reaction, while cut-off is defined as an accurate numerical value in ELISA. This difference in the scoring principles may have been responsible for the observed differences in sensitivities of the tests because only the samples with absorbeancies approaching the cut-off value were responsible for the lower sensitivity of ICT as compared with ELISA (82.2%). The sensitivity of ICT seems to be satisfactory for diagnostic purposes since it detected rotavirus at concentrations by three orders of magnitude lower than are those reported by Dar et al. (1994) for the initial stage of the infection. The agreement of results of ICT and Dot-ELISA (specificity 96.4%, sensitivity 97.8%) is due to the similar principles of results assessment.

In our experiments, ICT using rotavirus-specific IgY-antibodies bound to colloid gold was found to be a rapid and sufficiently sensitive method for the diagnosis of rotavirus-induced calf diarrhea. Considering the relatively easy preparation of IgY antibodies, the method meets the prerequisites for the development of a commercial diagnostic set.

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Information and advice for postgraduate students and new research workers. The completion of research projects by report only and disputation with reviewers and members of committee or board is only an exceptional approach which cannot replace the publication of results in peer-reviewed journals.

JAK HODNOTIT VÝSLEDKY VÝZKUMU: VÝZKUMNÉ ZPRÁVY A OPONENTNÍ ŘÍZENÍ

Oponentní projednávání má opodstatnění především u návrhů nových projektů a jejich metodik. Řešitelé musí k němu předložit jednoznačné návrhy a oponenti mají povinnost upozornit na jejich slabiny. Instituce poskytující finanční prostředky tak přenášejí odpovědnost za rozhodnutí alespoň částečně na posuzovatele a zvyšují pravděpodobnost účelného využití finančních prostředků. Riziko neúspěchu může být v tom, že řešitelé z obav před zneužitím svých nápadů nebo pro jejich nedostatek nepředloží dobré podklady k jednání anebo při řešení projektu nepostupují podle závěrů oponentního řízení. Stejně špatné je, když jsou oponenti nekompetentní, zaujatí nebo „nechtějí ublížit". Oponentní řízení není vhodné pro posuzování výsledků ukončených projektů a nemůže nahradit jejich uveřejnění v časopisech po úspěšném lektorském řízení. Důvodů je celá řada. Příprava podrobných zpráv zbytečně odvádí řešitele od přípravy publikací, ve kterých mohou být výsledky uveřejňovány průběžně podle jejich postupného získávání. Vyčkávání na dokončení všech částí projektu je nejen zbytečnou ztrátou času, ale přináší i nebezpečí, že na některé nedostatky bude upozorněno pozdě. Pokud se při oponentním řízení nějaké nedostatky zjistí, dostanou se v nejlepším případě do zápisu, často velmi opatrně vyjádřené, ale nemohou již ovlivnit průběh řešení. Nikdo z účastníků řízení nemá velký zájem na zjištění, že výsledky nelze přijmout. Tento závěr by jistě vadil pořádající instituci, která měla na problémy upozornit svými kontrolními mechanismy dříve. Nemusel by vadit oponentům, pokud se neobávají, že příště již nemusí být oponenty jmenováni. Ani pro odborně zdatné a odpovědné oponenty však není příjemné veřejně upozorňovat na vážné chyby v koncepci projektu a v metodice jeho řešení, které již nelze napravit. Omezí se proto často jen na formální připomínky ke zprávě a na návrhy k alternativám interpretace výsledků. Častým projevem opatrnosti oponenta a bezzubosti posudku je podrobná informace, kolik má zpráva stran, obrázků, grafů, tabulek a citací a jak krásně je vyvedena. Mimochodem, všimli jste si někdy, jak obtížně se na zprávy nebo projekty některých řešitelů hledají oponenti? Uzavření oponentního řízení usnesením, že výsledky nelze přijmout, by nepochybně nejvíc vadilo řešitelům. Žádné účastníky oponentního řízení proto nelze považovat za nezávislé. Pravděpodobnost přehlédnutí nebo záměrného zamlčení i závažných nedostatků je při oponentním řízení vyšší, než při lektorském řízení, kdy o posouzení rukopisů mohou být požádáni skutečně nezávislí a kompetentní lektoři třeba od protinožců. Anonymní lektor rukopisu určeného k publikaci je méně závislý než oponent. Jeho negativní stanovisko většinou porovnává redakční rada časopisu s názorem dalšího lektora a zkušení členové redakční rady jsou schopni odlišit malicherné nebo zaujaté připomínky od výhrad zásadních. Lektor má sice více možností ublížit neoprávněnými výtkami, ale i k výsledkům lektorského řízení se mají autoři možnost vyjádřit a nakonec mají možnost uveřejnit práci v jiném časopisu.

Při oponentním řízení se kamarádi mohou snažit ve veřejné diskusi autorům pomoci zdůrazňováním pracnosti a významu tématu a věhlasu řešitelů a „úhlavní přátelé" mohou naopak přikládat pod kotel z důvodů, známým často jen jim samotným. Za zamyšlení stojí i to, že téměř vždy se jen vzácně zúčastňují veřejných oponentních jednání hosté z vlastního zájmu. Je celkem lhostejné, zda příčinou je to, že nebyli informováni, natož pozváni, nebo zda jednání považují za ztrátu času. Vyjde to nastejno. Účastníky bývají pouze představitelé pořádající organizace, řešitelé, oponenti a členové oponentní komise nebo orgánu, jejichž formální hlasování je důležitější podmínkou úspěchu řízení než kvalita zprávy. Často je jednání z autorů přítomen jen odpovědný řešitel, protože spoluřešitelé buď nejsou přizváni nebo se neobtěžují svojí účastí. Někdy je jejich neúčast dokonce zdůvodňována ohromným pracovním vytížením. Oponentního řízení se však mají zúčastnit všichni řešitelé stejně jako přípravy zprávy nebo rukopisu publikace. Mají složit účty ze svojí činnosti a poučit se z průběhu jednání, aby se vyvarovali případných chyb a získali nové podněty k práci.

Co nebylo publikováno před oponentním jednáním, bude jenom výjimečně uveřejněno dodatečně. Financování projektu je ukončeno, řešitelé mají nebo by měli mít nové úkoly a povinnosti. Zpráva bývá obézní, protože tenká vyvolává podezření, že řešení nedalo mnoho práce. Obsahuje proto mnoho zbytečných informací, které zájemcům znesnadňují vyhledání dosažených výsledků a posouzení jejich kvality. Zpráva bude svázána do černého plátna, název projektu a jména autorů vytištěny zlatým písmem, u zvlášť pečlivých organizací se společně se zprávou sváží posudky, zápisy z oponentního jednání a stanovisko řešitelů s jejich poděkováním oponentům za pečlivé prostudování zprávy a za cenné připomínky. Zpráva se zařadí do fondu ústavní knihovny a odpovědný řešitel ji umístí na čestné místo v pracovně vedle desítek podobných, vlastních i těch, kterým pomohl na svět jako oponent. Zpráva se nedostane do referátových časopisů. Většinou se půjčuje pouze prezenčně a je proto obtížně dostupná případným zájemcům a zcela nedostupná v zahraničí. Při dnešních možnostech hledání informací v databázích na internetu a při možnosti sledovat řadu vynikajících časopisů on line nemá však náročný pracovník o zprávu ani zájem, protože údaje v ní uvedené stejně nemůže citovat. Proto se dobré výsledky, uváděné v závěrečných zprávách, nejčastěji dostávají do tisku nikoliv pod jménem svých autorů, ale pod jménem jiných, kteří je mohou beztrestně zcizit.

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DIFFERENTIATION OF VEROTOXIGENIC STRAINS OF *ESCHERICHIA COLI* ISOLATED FROM PIGLETS AND CALVES IN THE CZECH REPUBLIC*

DIFERENCIACE VEROTOXIGENNÍCH KMENŮ *ESCHERICHIA COLI* (VTEC) IZOLOVANÝCH ZE SELAT A TELAT V ČESKÉ REPUBLICE

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ABSTRACT: A set of 451 and 115 strains of *Escherichia coli* isolated from piglets and calves affected by diarrhoea, respectively, were tested for verotoxin production and type. The production was tested on Vero cells and the type was identified by PCR. The production of verotoxin was demonstrated in 189 of the porcine strains and 13 of the bovine strains. Most of the porcine verotoxigenic strains belonged to the serogroups O139, O141, O157, and O138. Positive for verotoxin production were 83.3% and 78.2% of the strains of the serogroups O139 and O141, respectively. The type VT2e was identified in 184 strains and a sequence corresponding to VT2c was found in 2 strains. However, the latter yielded negative results in the Vero cells test. Furthermore an unidentified toxin type was demonstrated in three strains positive in the Vero cell culture. The bovine verotoxigenic strains belonged to the serogroups O20, O26, O103, O111, and O157. Both VT1 and VT2 were produced by 6 (46.2%) of the 13 verotoxigenic strains. Only one verotoxin type was produced by each of the remaining verotoxigenic strains.

VT1; VT2; VT2e; VT2c; PCR; Vero cells; shigatoxins; verotoxins; Shiga-like toxins

ABSTRAKT: Produkce verotoxinů a jejich typ byl zjišťován u 451 kmene *Escherichia coli* izolovaného z nemocných selat a u 115 kmenů izolovaných z nemocných telat. Produkce byla sledována na buněčné linii Vero a typ toxinu byl určován PCR. Celkem bylo zjištěno 189 kmenů ze selat a 13 kmenů z telat produkujících verotoxiny. Produkce verotoxinu u kmenů ze selat byla nejčastěji prokázána v séroskupinách O139, O141, O157 a O138. Z kmenů séroskupiny O139 bylo 83,3 % a ze séroskupiny O141 bylo 78,2 % verotoxigenních. Ve 184 případech byl určen VT2e. U dvou kmenů byla nalezena sekvence odpovídající VT2c, vyšetření těchto dvou kmenů na buněčné linii Vero však dalo negativní výsledek. U tří kmenů, jejichž supernatanty vykazovaly cytotoxický efekt na Vero buňkách nebyl typ toxinu určen. U kmenů z telat byly verotoxigenní kmeny příslušné do séroskupin O20, O26, O103, O111 a O157. Z celkového počtu 13 verotoxigenních kmenů jich šest (46,2 %) produkovalo současně VT1 a VT2. Ostatní verotoxigenní kmeny produkovaly pouze jeden typ toxinu.

VT1; VT2; VT2e; VT2c; PCR; Vero buňky; shigatoxiny; verotoxiny; Shiga-like toxiny

INTRODUCTION

Escherichia coli is the causative agent of many animal and human diseases. The pathogenic strains differ from the nonpathogenic strains in virulence factors, including colonisation factors and toxins that are decisive for the pattern of the disease.

Verotoxigenic *E. coli* (VTEC) is a large group of strains with a characteristic cytotoxic effect on the Vero cell line (Konowalchuk et al., 1977). Verotoxins (VT) are produced by strains responsible for human diseases and animal diseases, of which oedema disease, affecting weaned piglets, is of particular importance. VT, the causative agent of oedema disease was earlier known as the oedema disease principle (EDP) (Clugston and Nielsen, 1974). Owing to their similarity with the toxin produced by *Shigella dysenteriae*, VTs are also designated as Shiga-like toxins (SLT) (O'Brien and LaVeck, 1983).

On the basis of biological effects, antigenic structure and heat sensitivity, VTs are divided into several types. The antigenic structure and biological properties of VT1 (SLTI) are almost identical with those of the toxin produced by *Shigella dysenteriae*. VT2 (SLTII) are divided into the subtypes VT2c (SLTIIc) and VT2e (SLTIIe). All the three differ from each other in genetic and antigenic properties (Strockbine et al., 1986). The causative agent of oedema disease of weaned piglets VT2e

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is neutralised by antibodies to VT2 (Marques et al., 1987) and shows a 92% homology in nucleotide and amino acid sequences with VT2 (Gyles et al., 1988). VT2e differs from VT2 in heat sensitivity (Gannon and Gyles, 1990). Considering the similarity between VT and toxins produced by shigellae the new terms Shiga-toxins (Stx1, Stx2, STx2c, Stx2e) and Shiga toxigenic *E. coli* (STEC) have also been suggested recently (Calderwood et al., 1996).

Most of the STEC strains isolated from piglets affected by oedema disease belong to the serogroups O138, O139, and O141. Their toxicity for Vero cells correlates well with their potential to induce lethal neurological signs in mice (Dobrescu, 1983). VT of these strains is a variant of Stx2 (Marques et al., 1987) now designated as Stx2e which is identical with EDP (VT2e, SLTIIe).

Most of the VTEC isolated from human patients are classified with the serotype O157 : H7, although other serotypes can also be involved (Karmali et al., 1985; Griffin, 1998; Caprioli and Tozzi, 1998). Human VTEC infections are caused by strains harboured often in the intestinal tract of cattle (Chapman et al., 1989), or other animals including the game (Chapman et al., 1989; Beutin et al., 1993; Chapman and Ackroyd, 1997; Sidjabat--Tambunan and Bensink, 1997). The occurrence of VTEC O157 in farm animals and rats in the Czech Republic was studied by Čížek et al. (1999).

The aim of our study was to identify verotoxigenic strains among animal *E. coli* isolates and to assess, on the basis of VT typing, their pathogenicity for animals and man.

MATERIAL AND METHODS

Strains

The *E. coli* strains H30 (O26 : H11, VT1+, VT2–), E32511 (0157 : H–, VT1–, VT2+), obtained from the Central Health Laboratory, London, and the strain 9030 (O139, VT2e+) were used as the prototype strains. The tested strains were isolated from sick or dying piglets and calves suspected of *E. coli* infections in 1980–1999. The strains were maintained in Dorset's egg slants and transferred on 5% ovine blood agar plates before testing. The set included 451 porcine and 115 bovine strains. The serotypes O157, O111, O26, O20, O115, and O116 of the bovine strains and the serotypes O138, O139, O141, and O157 of the porcine strains were selected for the testing.

Verotoxin detection

The tested strains were propagated in 100-ml vials containing 5 ml broth (Immuna, Šarišské Michalany, Slovakia) at 37 °C for 18 h under constant shaking. The cultures were treated with polymyxin (100 µg/ml) for 1 h and then centrifuged at 8 000 x g for 15 min. Penicillin (1 000 U/ml) and neomycin (1 000 µg/ml) were added to the supernatant to be tested for verotoxin. Vero cells were grown in wells of 96-well microtitre plates (NUNC, Camstrup, Denmark) containing 90 µl of MEM. Each well was inoculated with 30 µl of the supernatant. The results were read after 72 h of incubation at 37 °C and 5% carbon dioxide.

Verotoxin typing

VT were typed by polymerase chain reaction (PCR). The primer sequences are given in Tab. I. DNA of the tested strain was prepared by resuspension of one colony in 50 µl of deionised water and boiling for 15 min.

The initial cycle of 3 min at 94 °C, common to all the VT types, was followed by:

- 28 cycles of 92 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s for VT1;
- 28 cycles of 92 °C for 45 s, 63 °C for 45 s, and 72 °C for 70 s for VT2;
- 35 cycles of 92 °C for 45 s, 52 °C for 45 s, and 72 °C for 45 s for VT2c;
- 28 cycles of 92 °C for 45 s, 63 °C for 45 s, and 72 °C for 45 s for VT2e.

In the final phase of the reaction, the temperature of 72 °C was maintained for 3 min and the reaction mixture was cooled to 4 °C.

The amplification products were detected by electrophoresis in 2% agarose gel after staining with ethidium bromide and visualisation in a transilluminator.

I. Nucleotide sequences used as PCR primers

Primer	Nucleotide sequence	Amplification product	Reference
VT1 1	5-ACG-GCT-TAT-TGT-TGA-ACG-AA-3		Paton et al. (1995)
VT1 2	5-AAC-GAA-TGG-CGA-TTT-ATC-TG-3	204 bp	
VT2 1	5-GAC-AAC-GGA-CAG-CAG-TTA-TAC-C-3		Lin et al. (1993)
VT2 2	5-CCG-GAG-CCT-GAT-TCA-CAG-G-3	821bp	
VT2c 1	5-GCT-GGT-GTT-GTT-TTT-TTG-TG-3		Gannon et al. (1990)
VT2c 2	5-GTG-AGA-AAT-CGG-AGA-ATC-TGT-A-3	334bp	
VT2e 1	5-CGC-CGT-GAA-TGA-AGA-GAG-T-3		Weinstein et al. (1988)
VT2e 2	5-AAG-CCT-GAG-CCT-GAA-CTG-C-3	399bp	

RESULTS

As shown in Tab. II, most of the 189 porcine VTEC strains belonged to the serogroups O139 ($n = 83$; 83.3% positive) and O141 ($n = 44$; 78.2% positive). O-antigens of 26 verotoxigenic strains were not identifiable. Except for 5 strains, the verotoxins produced by the porcine strains were identified as VT2e. In 2 of the 5 strains, the nucleotide sequence corresponding to VT2c was identified, but tests in Vero cells yielded negative results. Supernatants of the remaining 3 (O141 and O?) induced cytotoxic effect in Vero cells, but their nucleotide sequence did not correspond to any of the four types considered within this study.

The results of detection and identification of verotoxins produced by the bovine strains of *E. coli* are shown in Tab. III. The production of verotoxin was demonstrated in 13 strains belonging to the serogroups O20, O26, O103, O111, and O157. Seven of the 13 verotoxigenic strains produced a single verotoxin type and 6 (46.2%) produced both VT1 and VT2.

II. Detection of verotoxins in porcine strains of *E. coli*

O-antigen	Number of tested strains	VT2e	VT2c	Unidentified VT	Test in Vero cells
O8	12				
O9	3	1			1
O35	7		1		
O45	6				
O51	2	1			1
O108	23	3	1		3
O111	6				
O115	1	1			1
O138	10	8			8
O139	99	83			83
O141	55	43		1	44
O145	1	1			1
O147	10	1			1
O149	69	2			2
O157	32	16			16
Other	17				
O?	98	24		2	26
Total	451	184	2	3	189

III. Detection of verotoxins in bovine strains of *E. coli*

O-antigen	Number of tested strains	VT1 + VT2	VT1	VT2	VT2c	Unidentified VT	VT total
O8	1						
O9	6						
O11	1						
O15	15						
O17	4						
O20	14	1				1	
O26	15			2	1		3
O86	3						
O103	1					1	1
O108	1						
O111	19	5	1		1		7
O115	5						
O116	4						
O117	4						
O119	1						
O157	4	1					1
O?	17						
Total	115	6	2	2	2	1	13

DISCUSSION

Results on porcine VTEC indicate that they covered a relatively wide range of serogroups. Most of them belonged to the serogroups O139, O138, and O141 which rank with the typical causative agents of oedema disease of weaned piglets (Bertschinger and Gyles, 1994). However, the production of verotoxin was demonstrated also in strains of serogroups that are not usually associated with oedema disease. VT2e, which is regarded as the causative factor of oedema disease, was identified in 184 of the 186 porcine verotoxigenic strains. The verotoxin(s) produced by 3 strains displaying cytotoxic effect in Vero cells differed from any of the four types considered within this study. The nucleotide sequence of two strains corresponded to VT2c, but its production was not confirmed by the test in Vero cells. The result was negative in one of them and dubious in the other.

VT2e was not detected in any of the 13 bovine verotoxigenic strains. Five and one strain producing both VT1 and VT2 belonged to the serogroups O111 and O157, respectively. Verotoxigenic strains of the serogroup O157 : H7 are generally considered the dominant group of VTEC causing human infections (Schmidt, 1999). In Europe, however, human infections are more often associated with other serogroups, in particular with O111 and O26 (Tozzi and Fisher, 1999). Diarrhoea in calves can be induced also by verotoxigenic strains of other serogroups, such as O103, that can be dangerous to human health, too (Dorn et al., 1993; Whipp et al., 1994).

Typing of verotoxins produced by animal strains of *E. coli* is necessary for the differentiation between dangerous human pathogens and strains that are pathogenic for animals only. Of particular importance is such typification in serogroups known to be associated with human diseases. A number of porcine strains of the serogroup O157 produce only enterotoxins (Alexa et al., 1995) and/or VT2e.

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THE COMPARISON OF MEBENDAZOLE AND FLUBENDAZOLE ANTHELMINTIC EFFICACY IN EXPERIMENTAL TREATMENT OF MOUFLON (*OVIS MUSIMON*) MUELLERIOSIS*

SROVNÁNÍ ANTHELMINTICKÉ ÚČINNOSTI MEBENDAZOLU A FLUBENDAZOLU PŘI EXPERIMENTÁLNÍ LÉČBĚ MUELLERIÓZY MUFLONÍ ZVĚŘE (*OVIS MUSIMON*)

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ABSTRACT: The mebendazole and flubendazole efficacy (identical dosage scheme 7.5 mg/kg of body weight/day, 3 consecutive days) in treatment of mouflon muelleriosis was studied. Parasitical parameters (larval excretion in fecal samples, larval and worm egg presence in lung verminous tissue of shot animals) were determined. The post-treatment level of helminthological findings was different, generally higher anthelmintic efficacy was found with flubendazole.

Ovis musimon; *Muellerius capillaris*; benzimidazoles

ABSTRAKT: Výsledky ověření účinnosti mebendazolu v léčbě muelleriózy mufloní zvěře byly jinými autory opakovaně vyhodnoceny jako neuspokojivé (Páv aj., 1973; Špenik a Halasz, 1978; Chroust, 1982; Švandová, 1987). Naproti tomu naše zkušenosti s využitím flubendazolu ve shodné indikaci byly vysoce pozitivní (Lamka aj., 1996b). Tento rozpor, tj. vysoká chemická podobnost zmíněných léčiv (flubendazol je fluór analogem mebendazolu) a velmi různorodé experimentální zkušenosti, nás přivedl k uskutečnění dvou studií. Jejich cílem bylo porovnat účinnost obou léčiv proti *Muellerius capillaris* přirozeně infikované mufloní zvěře. Z celooborní populace byly náhodným výběrem vyčleněny dvě skupiny zvěře. Tyto byly identické počtem kusů ($n = 6$), pohlavím i věkem (poměr muflončat a muflonek). Zvěř experimentálních skupin byla chována zvlášť (asi 3 ha oplocené plochy), ale shodně byl zajišťován sběr směsných vzorků trusu před, v průběhu a po podání testovaných léčiv v denní dávce 7,5 mg/kg ž. hm. tři dny po sobě. Hmotnost živé zvěře byla před podáním léčiv odhadnuta. Na konci ověření byla zvěř odlovena, zvážena a podrobena detailní helmintologické pitvě (makroskopické vyšetření plic, odběr verminózní tkáně plic, odběr individuálních vzorků trusu). Všechny biologické vzorky byly opakovaně larvoskopicky vyšetřeny na přítomnost L_1 larev (směsné a individuální vzorky trusu) i L_1 larev a vajíček (verminózní tkáně plic po jejím jemném nastříhání) Baermannovou metodou. Ve vzorcích trusu byl stanoven počet larev v 1 g vyšetřovaného materiálu (LPG). Studie byly uskutečněny v zimních měsících roku 1998, resp. 1999. Předléčebná úroveň parazitace jednotlivých experimentálních skupin zvěře byla rozdílná (tab. I). Také skutečně použitá dávkovací schémata testovaných léčiv nebyla vždy v souladu se zamýšleným dávkováním (tab. II). Koprologické nálezy ve vzorcích směsného trusu (rok 1998) po léčbě velmi rychle poklesly až k negativním nálezům (častěji u mebendazolové skupiny) (obr. 1). Helmintologické nálezy v plicích byly v celém sledovaném rozsahu nižší u flubendazolové skupiny (tab. III). Následující studie (rok 1999) byla metodicky rozšířena. Bezprostřední poléčebné nálezy LPG byly při porovnání testovaných léčiv opět shodné a průběhem podobné roku 1998 (obr. 2), v pozdějších časových intervalech byl ale prokázán opětovný návrat exkrece L_1 larev. Ten byl průběhem již individuální, strmější u mebendazolu. Pro porovnání účinnosti sledovaných anthelmintik byly stanoveny plochy pod koprologickými křivkami, jejich poměr mebendazol/flubendazol je 1,62. Pitevní helmintologické nálezy byly u obou skupin vyrovnanější než v první studii. Vzhledem k výsledkům dříve uveřejněných studií byla účinnost mebendazolu vyhodnocena jako neočekávaná. Vysvětlení lze najít jednak v našem detailněji vedeném ověření, jednak v použití modernější léčivé substance mebendazolu (polymorph-C) mající příznivější farmakokinetické parametry (Wesche a Barnish, 1994 výsledky Janssen Research Foundation, 1998 – nepublikováno). Výsledky lze celkově shrnout konstatováním, že při přihlédnutí k použitému dávkování a úrovni předléčebné parazitace jednotlivých skupin zvěře byla prokázána vyšší anthelmintická účinnost v léčbě muelleriózy mufloní zvěře u flubendazolu.

Ovis musimon; *Muellerius capillaris*; mebendazol; flubendazol

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INTRODUCTION

Nematodoses are the most frequent helminthoses in cloven-hoofed game. Many drugs have been used to control nematodoses and have good efficacy against gastrointestinal nematodes and large lungworms. Currently, the group of small lungworms (*Protostrongylidae*) remains a therapeutical problem, even though the importance of its pathogenicity in game is well known. The *Muellerius capillaris* infection in mouflon is a typical example. The effective therapeutical approaches were described in our earlier papers (Lamka et al., 1996a, b, 1997). Flubendazole administration caused fully effective control of *M. capillaris*. Similar therapeutical schemes resulted in the same indication when using mebendazole (Páv et al., 1973; Špenik and Halasz, 1978; Chroust, 1982; Švandová, 1987) in limited or zero efficacy. This contradiction, which is the chemical relation of these drugs (flubendazole is a fluoro analogue of mebendazole) and the very different experimental findings, led us to focus on these experimental studies.

MATERIAL AND METHODS

General arrangement of studies

Two experimental groups of animals naturally infected by *M. capillaris* were randomly selected from the mouflon population of game park. Then the groups were placed into separate enclosed areas of about 3–4 hectares during the studies. Both groups of mouflons were identical in the number of animals ($n = 6$), sex (4 females, 2 males), and age (2 mouflon ewes, and 4 lambs). The simultaneous collections of feces, administrations of tested drugs, parasitological evaluations and mouflon shot were conducted. The studies were performed in the winter season from January to March 1998 and 1999.

Drug administration

Mebendazole (Telmin granulate, Janssen Pharmaceutica) and flubendazole (Flubenol 50% premix, Janssen Pharmaceutica) were homogeneously mixed with standard mouflon feed. The daily dose of 7.5 mg/kg of body weight in 3 consecutive days (dosage scheme 3 x 7.5 mg/kg of body weight) was used. The total live body weights of individual mouflon groups before the administrations of the drugs were approximated. Medicated feed was administered daily, the feed intake was controlled as well. The exact body weights of mouflons were determined after the study termination and the corresponding exact dosing of drugs was calculated.

Fecal samples and necropsy materials

The group fecal collections were taken prior to (on days 1–5 of the studies), during, and after the experimental treatment. Samples of feces were examined for larvae by Baermann method. The number of larvae per 1g of fecal material (LPG) was determined. All hunted animals were subjected to detail helminthological examinations. Lungs of individual mouflons were macroscopically evaluated, verminous spots were removed, gently cut and examined for the presence or absence of L_1 live larvae or worm eggs (Baermann method). Individual fecal samples were examined larvoscopically as well. Biological samples (3 g) were regularly used, all evaluations were conducted repeatedly.

Mathematical data evaluation

All numerical values presented in tables and figures are mean values. The areas under the coprological curves (AUCC) also were calculated.

RESULTS

LPG pretreatment values and drug dosing

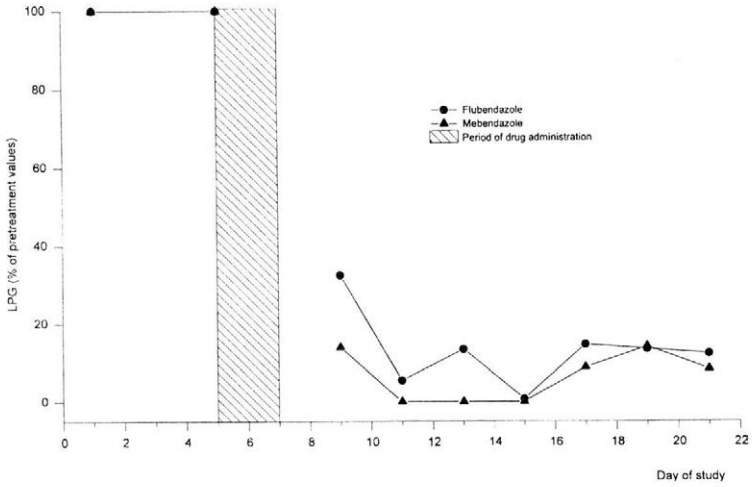
The mean pretreatment LPG values in pairs of experimental groups were not equal especially in 1999 (Tab. I). Actual drug dosing was slightly higher than the proposed one (Tab. II), larger differences between tested benzimidazoles was calculated in 1998.

I. Pretreatment LPG values in mouflon fecal samples

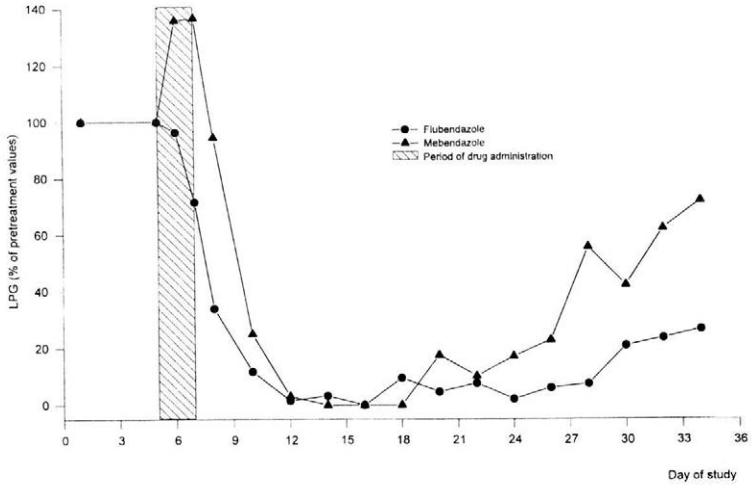
Tested drug	Number of evaluations	LPG	
		1998	1999
Mebendazole	24	637	417
Flubendazole	24	415	2 596

II. Proposed and real dosage schemes of tested drugs

Tested drug	Year of study	Proposed drug dosage scheme (mg/kg of body weight)	Real drug dosage scheme (mg/kg of body weight)
Mebendazole	1998	3 x 7.5	3 x 8.9
	1999	3 x 7.5	3 x 7.5
Flubendazole	1998	3 x 7.5	3 x 7.8
	1999	3 x 7.5	3 x 8.1



1. LPG values of *M. capillaris* after mebendazole and flubendazole administration in mouflons (year 1998)



2. LPG values of *M. capillaris* after mebendazole and flubendazole administration in mouflons (year 1999)

LPG in group fecal samples

A significant and rapid decrease in L_1 larval excretion was shown with both studies (more frequently in the mebendazole groups), during the later time intervals (1999) LPG values gradually and drug-individually increased (Figs. 1, 2). Mebendazole AUCC value (1447.4 percentdays) is higher than the flubendazole value (893.7 percentdays).

Helminthological findings in lungs and feces of necropsied animals

Due to the *M. capillaris* infection in all of the studied animals, macroscopically visible lung verminous spots were identified. The range of verminous damage individually varied from ten minute spots to several diffuse large foci. The L_1 larval and egg presence in the verminous lesions were found in some of the tested samples (Tab. III). We can summarize all necropsy findings (after the comparison of experimental groups in both studies) that in most of the evaluated parameters a lower intensity of post-treatment parasitism was found in flubendazole groups.

DISCUSSION

The flubendazole helminthocidal effect in mouflon muelleriosis was documented in a 3 x 15 mg/kg of body weight dosage scheme (Lamka et al., 1996b). The exact anthelmintic scheme was not suitable for these compara-

III. Helminthological findings in shot mouflons

Tested drug	Year of study	Prevalence of verminous spots (%)	Prevalence of L_1 larvae in verminous spots (%)	Prevalence of worm eggs in verminous spots (%)	Prevalence of L_1 larvae in feces (%)	Mean LPG value in feces
Mebendazole	1998	100	70.0	36.7	66.7	53 (n = 30)
	1999	100	100.0	60.7	50.0	100 (n = 18)
Flubendazole	1998	100	53.0	23.3	33.3	51 (n = 30)
	1999	100	82.0	67.8	66.7	230 (n = 18)

n = number of examinations

tive studies, therefore the reduced dosing (3 x 7.5 mg/kg of body weight) for both tested drugs was proposed. The initial study in 1998 was aimed to verify the proposed scheme from an anthelmintic point of view. The absolutely significant and time limited decreases in larval excretion were positively evaluated. However, the same study was too short to give us a possibility to describe interesting later LPG developments. Among the other important findings of this study, flubendazole had better necropsy results than mebendazole.

The results in 1999 fulfilled our expectations concerning the comparison of anthelmintic efficacies. The standard decrease in larval excretion is followed by different trends in larval coprological activity returns. The explanation of these facts can be found in individual anthelmintic potentials (helminthostatic or partially helminthocidal effects) of tested benzimidazoles against *M. capillaris* larvae and adults. Higher flubendazole efficacy is apparent, AUCCs share (mebendazole/flubendazole) 1.62 expresses the ratio of mutual efficacy. The findings of helminthological necropsy do not fully confirm LPG values especially during the days of study termination. All results, however, were reached under very different levels of group pretreatment parasitism. Finally, it is possible to characterize the results of the second study as the enlarging and supporting results of initial study.

Mebendazole anthelmintic activity found in these studies is contradictory to earlier experimental findings and can be expressed as unexpected. Two reasons may be responsible for this contrast. First, different methodological approaches were used (much more detailed LPG values determinations were used in our experiments), second, active substance was different. Former studies used an older type of mebendazole substance, we have administered a modern polymorph-C type characterized by better pharmacokinetics (Wesche and Barnish, 1994; unpublished results of Janssen Research Foundation, 1998). Based on the described results, it is possible to identify flubendazole as a more effective anthelmintic of mouflon muelleriosis.

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THE USE OF EGG YOLK IMMUNOGLOBULIN IN THE DIAGNOSTICS OF CANINE PARVOVIRUS INFECTIONS*

VYUŽITÍ IMUNOGLOBULÍNU VAJEČNÉHO ŽLOUTKU K DIAGNOSTICE PARVOVIROZY PSŮ

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ABSTRACT: ELISA procedures for the detection of canine parvovirus-2 (CPV-2) in faecal samples and of CPV antibodies in blood serum or whole blood are described. The basic component of the two procedures is solid phase-bound egg yolk antibodies to CPV-2 – IgY. Comparative trials of ELISA and haemagglutination test using 96 faecal samples demonstrated a 75% agreement of results. A comparison of ELISA and haemagglutination inhibition test using 149 canine blood serum samples yielded agreeing results in 87.9% of the animals. The usability of ELISA for the demonstration of antibodies to CPV-2 in whole blood was tested by examination of 70 and 36 samples collected from adult dogs and vaccinated pups, respectively. The differences between the blood serum and whole blood titres were insignificant ($p > 0.05$) and their correlation was expressed by the coefficient $r = 0.870$.

egg IgY; ELISA; haemagglutination; haemagglutination inhibition; canine parvovirus-2; antigen; antibodies

ABSTRAKT: Pro diagnostiku parvovirózy psů byly na principu enzymoimunoanalýzy (ELISA) vyvinuty metody průkazu viru CPV-2 ve fécés a protilátek v krevním séru, resp. v krvi. Základní komponentou obou testů byly specifické anti-CPV-2 vaječné protilátky – IgY, které byly vázány na pevný nosič. Izolace IgY dosahovala výtěžnost asi 150 mg na jeden žloutek s ELISA titrem anti-CPV-2 protilátek až 100 000. Pro srovnání ELISA s hemaglutinačním testem bylo použito 96 vzorků fécés. Shoda ve výsledcích byla zjištěna 75% (tab. I). Spolehlivost ELISA pro průkaz protilátek byla ověřena vyšetřením 149 krevních sér. Srovnáním s testem inhibice hemaglutinace byla dosažena shoda 87,9 % (tab. II) a korelační koeficient 0,745. Využitelnost ELISA průkazu protilátek v celé krvi odebírané do mikrokapilár byla potvrzena vyšetřením 70 vzorků odebraných od psů (tab. IV) a 36 odebraných od vakcinovaných štěňat. Korelaci výsledků dosahovaných ELISA v krevních sérech a v celé krvi vyjadřuje koeficient 0,870.

vaječný IgY; ELISA; hemaglutinace; inhibice hemaglutinace; CPV-2; antigen; protilátky

INTRODUCTION

Canine parvovirus infection, defined as a nosological entity in 1978 (Appel et al., 1979), is a world wide occurring contagious disease (Mathys et al., 1983). On the basis of its morphological, structural and biological characteristics, the causative agent isolated from faecal samples of dogs suffering from haemorrhagic enteritis has been classified as parvovirus Type 2 (CPV-2) (Appel et al., 1979; Carpenter et al., 1980; Moraillon, 1994; Svoboda et al., 1994).

Numerous methods, such as virus isolation, electron microscopy, haemagglutination, virus neutralization, haemagglutination inhibition, immunofluorescence, latex agglutination, and radial haemolysis tests, and polymerase chain reaction, have been suggested as diagnostic methods for parvovirus infections (Arens and Kraus, 1980; Carmichael et al., 1980; Carmichael and Binn, 1981; Mathys et al., 1983; Appel and Parrish, 1987; Senda et al., 1988; Uwatoko et al., 1995).

The above methods have been completed by various modifications of ELISA for the demonstration of antigen or antibodies. The accuracy of ELISA results depends on the origin and purity of components, in particular antibodies, used in the assay. Antibodies obtained from blood sera of immunized laboratory animals (guinea pigs, rabbits), or monoclonal antibodies are used for this purpose in most cases. Antibodies to parvovirus of this origin were used in the development of ELISA techniques described, among others, by Mildbrand et al. (1984) and Drane et al. (1994).

Authors of recent papers have suggested, however, that antibodies obtained from phylogenetically lower animal species should be preferred in the construction of enzymoimmunoanalytical tests for the diagnostics of mammalian infectious diseases (Shade et al., 1997). Such

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antibodies can be obtained from laying hens in which serum immunoglobulins pass into developing eggs and concentrate in egg yolk (Shade and Hlinak, 1996). Yolk antibodies, designated as IgY, are the source of passive immunity for chicks. The specificity of IgY can be modified by active immunization of hens (Schmidt et al., 1989; Gassman et al., 1990; McLaren et al., 1994; Hlinak et al., 1996). IgY can be isolated from egg yolk by relatively simple procedures (Jensenius et al., 1981; Hatta et al., 1990; Akita and Nakai, 1993).

The objective of our experiment was to test the usability of antibodies obtained from egg yolks of hens immunized with canine parvovirus CPV-2 as a component of ELISA for the diagnosis of canine parvovirus infection.

MATERIAL AND METHODS

Antigens

The CPV-2 strain OP-1/81, supplied by Opavet, Ltd., Opava, Czech Republic, was propagated in the established cell line FE (Carmichael et al., 1980) grown in Eagle MEM supplemented with 5% bovine foetal serum. Two millilitres of a virus suspension with haemagglutination titre 1 : 256 were inoculated into cell suspensions with a density of 2 x 10^5 cells per 1 ml of growth medium immediately after seeding. The growth medium was replaced by maintenance medium (Eagle MEM without calf serum) 24 to 48 h after the inoculation. Two to four days later, the cell cultures were frozen-thawed once and cell debris was removed by low-speed centrifugation at 7 000 r.p.m. for 20 min in the centrifuge L2-21, rotor 7.5 (Beckman Instruments, Palo Alto, USA).

The supernatant (viral suspension) was inactivated with 0.15% β-propionlactone and used as a positive control antigen in ELISA. Negative control antigen was prepared by the above procedure using mock-infected cell cultures.

The pellets obtained by centrifugation of the viral suspension at 4 °C and 100 000xg for 2 h (L-8 80M, rotor 45 Ti, Beckman Instruments) were resuspended in PBS, pH 7.2, tested by ELISA and haemagglutination test and used as inocula.

Antibodies

Two parts of resuspended viral proteins were mixed with one part of complete (1st immunization) or incomplete (subsequent immunizations) Freund adjuvant. Californian rabbits aged 3 months were immunized subcutaneously 3 times at intervals of 21 days with 1 ml of the inoculum distributed into four administration sites. The rabbits were sacrificed and bled one week after the 3rd immunization and antibodies were precipitated from blood sera using the standard procedure with ammonium sulphate.

Hens that were immunized with 0.5 ml of the viral proteins repeatedly at 14-day intervals. Parvovirus-specific antibodies were purified from yolks of eggs collected starting from day 50 of the immunization period.

IgY were isolated from yolk suspensions using the procedure described by Jensenius et al. (1981). Briefly, this included the removal of lipids with dextran sulphate and precipitation with 40% ammonium sulphate (Sigma, St. Louis, USA).

The contents of specific antibodies were determined by double sandwich ELISA which was similar to the procedure for the detection of canine antibodies to CPV-2 (see below). Capture antibody was rabbit IgG to CPV-2 at the concentration of 6 µg/ml, and swine antibody to chicken IgG labelled with horse radish peroxidase by the periodate method (Farr and Nakane, 1981) was used as the conjugate. CPV antigen and antibodies were detected by haemagglutination (HT) and haemagglutination inhibition (HIT) tests as described by Appel et al. (1979) and Carmichael et al. (1980).

Blood and faecal sample processing

Blood and faecal samples were obtained from two kennels of police dogs, several veterinary practitioners, and the University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic.

A set of 149 blood samples collected from adult dogs and pups was available. Twelve pups were sampled before and after the 1st and 2nd vaccinations, i. e. at the age of 9, 12 and 15 weeks. Standard procedures were used for sampling and sample processing. Microvolume (10 µl) whole blood samples were simultaneously collected into heparinized capillaries from 70 adult dogs and all the vaccinated pups (Rodák et al., 1985). Blood was rinsed out of the capillaries into 1 ml of diluting solution immediately after withdrawal and used for the determination of antibodies by ELISA without further processing.

Faecal samples were collected from 34 healthy dogs and pups and 62 animals showing signs of enteritis. The samples were suspended in PBS, pH 7.2, centrifuged at 4 000 r.p.m. for 15 min and stored at –20 °C.

ELISA for parvovirus detection

CPV-2-specific IgYs at the concentration of 50 µg per 1 ml of the coating buffer (0.05 M sodium carbonate buffer, pH 9.6) were bound to the wells of microtitre plates (Nunc, Maxisorp, Roskilde, Denmark) overnight at 4 °C. Twofold dilutions of faecal samples were applied into the wells and incubated at 37 °C for 60 min. Positive and negative controls were run in each plate. IgY antibody to CPV labelled with horse radish peroxidase (Sigma, St. Louis, USA) using the periodate method (Farr and Nakane, 1981) was added. Each step of the procedure was followed by washing of the plates with PBS, pH 7.2. After another hour of incubation, hydrogen peroxide + tetramethyl benzidine (Sigma, St.

Louis, USA) was added as the substrate. The colour reaction was stopped with 1M sulphuric acid after 10 min of incubation at room temperature. Optical density was read spectrophotometrically at 450 nm (SLT Spectra, Schoeller, Austria). Samples showing absorbances > 0.200 were scored as positive.

ELISA for CPV-2 antibody detection

CVP-2-specific IgY diluted with carbonate buffer, pH 9.6, to a concentration of 50 µg/ml was bound to microtitre plates overnight at 4 °C. Viral and negative control antigens diluted 1 : 4 were then applied alternately into individual columns. After 60 min of incubation at 37 °C, serial sera or blood dilutions were tested, starting with 1 : 100. Positive and negative control sera were included in each plate. After incubation at 37 °C, a conjugate, i. e. antibodies to canine or rabbit IgG prepared by affinity chromatography and labelled with horse radish peroxidase (Sigma, St. Louis, USA) was added into the wells. Each step of the procedure was followed by 3 washes of PBS, pH 7.2. Teramethyl benzidine + H_2O_2 was used as the chromogenic substrate. The plates were incubated at room temperature for 10 min and the colour reaction was stopped by the addition of 1M sulphuric acid. The optical density values were measured spectrophotometrically at 450 nm (SLT Spectra, Schoeller, Austria).

Samples showing absorbances with the positive control antigen higher than 0.1 were scored as positive provided that the difference between the absorbances with the positive and the negative antigens was higher than 0.1.

IgY antibodies were replaced by rabbit antibodies and antibodies to anti-canine IgG in the conjugate by antibodies to IgY to detect egg yolk antibodies to parvovirus.

RESULTS

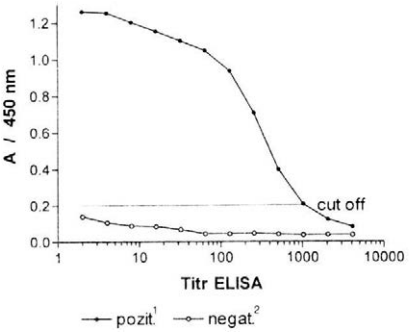
Detection of IgY to CPV-2 in eggs of inoculated hens

Egg yolks from inoculated hens yielded the concentration of 10 mg IgY per 1 ml. At the mean yolk volume of 15 ml, the per egg yield was 150 ± 20 mg. The antibody titre as determined by ELISA, ranged between 50 000 and 100 000. Current checks demonstrated that the purified IgYs could be stored for up to 2 years at –20 °C without the significant loss of titre.

Use of IgY to CPV-2 in ELISA

Detection of CPV-2 antigen

The evaluation of CPV-2 detection by ELISA was performed after the assessment of ELISA cut-off values using the positive and negative faecal samples (Fig. 1). Based on testing the population of known negative and positive samples, the cut-off value of 0.2 was selected. This value was not exceeded by any of the negative samples. Numerically, the cut-off titre value was 1 024. The characteristic of the assay was shown by the sigmoid shape of the curves expressing the relation between absorbance and antigen concentrations.



1. Titration curves of CPV in control samples of dog faeces ELISA titre

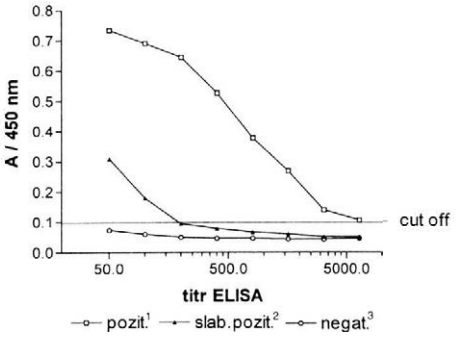
[1]positive, [2]negative

I. Comparison of HT and ELISA for detection of CPV-2

Haemagglutination		ELISA	
Titre	*n*	positive	negative
0	26	0	26
4–128	40	20	20
256	6	4	2
512	8	8	0
≥1 024	16	16	0
Total	96	48	48

The newly developed ELISA was further validated by testing the 96 faecal samples collected from clinically healthy dogs and pups and those showing the clinical signs of enteritis. Parvovirus was detected in 48 faecal samples collected from the latter category. Virus ELISA titres ranged between 128 and 2 048. Only 3 and 2 samples showed lower (4–16) and higher (up to 8 192) titres, respectively.

ELISA and HT were compared for 5 categories of the samples as determined by HT (Tab. I). 100% agreement was found only for the samples with HT titres > 256. Some samples with the lower HT titres were negative by ELISA. This apparently lower sensitivity of ELISA (68.9%) resulted from the higher number of HT-positive samples including also those yielding false positive results caused by nonspecific agglutinins or other enteric haemagglutinating agents (Drane et al., 1994). Unlike HT, ELISA was negative in all the clinically healthy dogs. None of the HT-negative samples were positive by ELISA.



2. Titration curves of anti-CPV antibodies in control blood sera of dogs

[1]positive, [2]weakly positive, [3]negative

II. Comparison of HIT and ELISA for antibodies to CPV-2

Haemagglutination inhibition		ELISA	
titre	*n*	positive	negative
0	40	14	26
4–128	32	28	4
256	17	17	0
512	22	22	0
1 024	19	19	0
2 048	15	15	0
4 096	4	4	0
Total	149	119	30

III. Titres of antibodies to CPV-2 reacting in ELISA and haemagglutination inhibition test

ELISA titre	*n*	HIT titre
0	3	17 ± 43
50	12	85 ± 60
100	15	120 ± 56
200	12	85 ± 150
400	9	256 ± 147
800	11	500 ± 280
1 600	10	371 ± 145
3 200	17	888 ± 602
6 400	13	1181 ± 1006
12 800	14	1826 ± 1037
25 600	6	2389 ± 763
Total	149	

Detection of IgG antibodies to CPV-2

ELISA for detection of antibodies to canine parvovirus was evaluated after setting the cut-off values using the positive, weakly positive, and negative blood serum samples. Based on testing the population of known negative and positive samples, the cut-off value of 0.1 was set. Numerically, the cut-off values for the standard positive and weakly positive sera were 6 400 and 200, respectively. The titration curves are presented in Fig. 2. Antibodies to CPV-2 were demonstrated in 79.9% (119 of 149) of the blood serum samples. A 87.9% accordance was found for the results of ELISA and HIT (Tab. II) and 100% accordance was found for the samples with HIT titres > 128. Marked differences between the results of the two methods were observed in samples with lower or negative HIT titres; in the latter category, 14 of the 40 samples were positive by ELISA. The correlation between the results of HIT and ELISA was expressed by the coefficient $r = 0.745$, $p > 0.05$ (Tab. III).

The application of ELISA for examination of whole blood samples was tested by simultaneous examination of blood samples collected into microcapillaries and blood serum samples. The whole blood titres were lower than the blood serum titres, but the difference was irrelevant (Tab. IV). The correlation between the results of the two variants was expressed by the coefficient $r = 0.870$, $p > 0.05$.

IV. Comparison of blood serum and whole blood titres of antibodies to CPV-2

Blood serum titre	*n*	Whole blood titre
0	5	0
100	5	100 ± 63
200	5	100 ± 63
400	5	320 ± 98
800	6	500 ± 223
1 600	13	1292 ± 389
3 200	16	1950 ± 746
6 400	10	3840 ± 1280
12 800	5	7040 ± 3135
Total	70	

V. ELISA titres of antibodies in whole blood of pups before and after vaccination

Age(weeks)	9	12	15
Pup No.	before vaccination	before revaccination	after revaccination
1	200	25 600	25 600
2	0	25 600	25 600
3	0	25 600	25 600
4	100	6 400	6 400
5	200	100	12 800
6	200	12 800	25 600
7	800	25 600	25 600
8	6 400	12 800	12 800
9	200	6 400	6 400
10	100	12 800	25 600
11	400	6 400	25 600
12	100	3 200	12 800

The reliability of antibody detection in whole blood samples was further tested by examination of 36 blood samples collected from vaccinated pups (Tab. V). While the pre-vaccination titres at the age of 9 weeks ranged from zero to 6 400, the vaccination and re-vaccination resulted in an unambiguous increase in all the pups reaching the range from 6 400 to 25 600. The difference between the mean pre-vaccination and post-vaccination titres was significant ($p = 0.01$).

DISCUSSION

The importance of the IgY technology in biomedical research has been growing in recent years (Schade et al., 1997). One of the reasons stipulating this development is the increasing interest of the public in the protection of experimental animals. The IgY technology is a natural way how to meet the requirements of the public and to obtain a permanent source of large quantities of antibodies that can be used both in diagnostics (Schmidt et al., 1989; Chandan et al., 1994; Hlinak et al., 1996) and in passive immunization of farm and companion animals (Bartz et al., 1980; Yolken et al., 1988; Wiedemann et al., 1991; Kuroki et al., 1994). Further reasons for the implementation of the IgY technology are low production costs and simple processing methods.

This paper describes the preparation of IgY with antibody activity to CPV-2. The per egg yield of 150 mg of IgY was in agreement with data published by other authors, i.e. 50 to 100 mg (Schade and Hlinak, 1996; Schwarzkopf and Thiele, 1996). The obtained antibodies were used in ELISA for the detection of the antigen and antibodies to CPV-2 with the aim to replace HT and HIT or prepare an alternative test. The HT and HIT are extensively used by diagnostic laboratories despite of the problems with variable characteristics of erythrocytes, occurrence of non-specific haemagglutinins and non-specific inhibitors of haemagglutination, and difficulties with applications of semiautomatic procedures (Mathys et al., 1983; Drane et al., 1994). The reliability of HT and HIT was lower, particularly at lower dilutions of the tested faecal and blood serum samples, resulting in a lower percentage of accordance with the results of ELISA (75 and 88% for HT and HIT, respectively). Compared with HT, the sensitivity and specificity of ELISA was 68.6% (48 of 70 samples) and 100% (26 of 26 samples), respectively. Compared with HIT, the calculated sensitivity and specificity was 96.3% (105 of 109 samples) and 65% (26 of 40 samples), respectively. While the results of antibody titration by ELISA and HIT correlated at $r = 0.745$, no correlation was found for the results of antigen titration by ELISA and HT, the accordance being limited only to negativity and positivity of the results. One of the apparent reasons of this failure was the poor reproducibility of the results of titration by HT.

Attempts to improve the diagnostics of canine parvovirus infections were an impetus of several laboratories for the application of sandwich or competitive ELISAs (Mathys et al., 1983; Mildbrand et al., 1984; Rimmelzwann et al., 1990; Burtonboy et al., 1991; Drane et al., 1994). In our experiments, sandwich ELISA with polyclonal capture IgY antibodies to CPV-2 bound to a solid carrier was used to simplify the ELISA procedure without affecting its sensitivity and specificity. IgY allowed us to avoid the use of monoclonal antibodies and reduce the amounts of CPV-2 antigens necessary for direct antigen binding. The species difference between the chicken and mammalian immunoglobulins might reduce the occurrence of cross reactions and unwanted background values (Schade and Hlinak, 1996). Moreover, IgY does not possess the Fc-receptor and therefore activates neither the complement system nor other factors (Larson et al., 1993; Rieger et al., 1996).

A method of whole blood sampling into microcapillaries, intended only for ELISA, was developed by Rodák et al. (1985). Although the sensitivity of assays with whole blood samples was slightly lower, comparative tests showed a satisfactory correlation with the results of blood serum testing. Compared with whole blood testing, the sensitivity and specificity of blood serum ELISA was 96.9% and 100% ($p > 0.05$), respectively. Irrespective of the fact that the tests were done in pups with different initial levels of immunity, it is evident that whole blood testing is a feasible method for checking the vaccination effect in pups without causing excessive stress to the vaccinates. It can be concluded that the use of IgY in ELISA for the diagnosis of canine parvovirus infections, combined with whole blood sampling, meets the requirements for ethical treatment of experimental animals without affecting the reliability of results.

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BIOCHEMICAL MONITORING OF AQUATIC POLLUTION: INDICATORS OF DIOXIN-LIKE TOXICITY AND OXIDATIVE STRESS IN THE ROACH (*RUTILUS RUTILUS*) AND CHUB (*LEUCISCUS CEPHALUS*) IN THE SKALICE RIVER *

BIOCHEMICKÉ MONITOROVÁNÍ ZNEČIŠTĚNÍ VODY: INDIKÁTORY TOXICITY DIOXINOVÉHO TYPU A OXIDATIVNÍHO STRESU V PLOTICI OBECNÉ (*RITULUS RUTILUS*) A JELCI TLOUŠŤOVI (*LEUCISCUS CEPHALUS*) V ŘECE SKALICI

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ABSTRACT: Concentrations of major classes of organic contaminants were determined in muscle samples and biochemical markers indicating dioxin-like toxicity and oxidative stress were measured in liver samples collected from roach (*Rutilus rutilus*) and chub (*Leuciscus cephalus*) fished out at nine sites of the Skalice river (Czech Republic). No significant differences were found in the concentrations of polychlorinated biphenyls (PCBs) and organochlorine insecticides between the two species. The concentrations of polycyclic aromatic hydrocarbons were higher in roach than in chub. Point sources of PCBs as the major environmental contaminant class were identified. The data on PCB concentrations in muscle tissue correlated well with the induction of the CYP1A-dependent 7-ethoxyresorufin *O*-deethylase activity (EROD) as the biomarker of the dioxin-like toxicity in the hepatic microsomal fraction of roach and chub. Changes in the hepatic microsomal glutathione *S*-transferase and cytosolic glutathione reductase activities were used as selective biomarkers of oxidative stress. The modulations of the two enzymes by environmental contaminants were almost identical, but differed markedly from the pattern of EROD induction correlating rather with concentrations of lindane and DDT incl. its metabolites than with those of PCBs.

biomarkers; PCBs; organochlorines; PAHs; CYP1A; dioxin toxicity; oxidative stress

ABSTRAKT: Ve svalovině dvou sladkovodních druhů ryb, plotici obecné (*Rutilus rutilus*) a jelci tloušťovi (*Leuciscus cephalus*), odchycených v devíti lokalitách řeky Skalice (Česká republika) byly stanoveny koncentrace hlavních tříd organických kontaminantů; zároveň byly měřeny v jaterní tkáni těchto ryb biochemické markery detekující toxicitu dioxinového typu a oxidativní stres. Akumulace polychlorovaných bifenylů (PCB) a organochlorových pesticidů ve svalové tkáni byla podobná v obou druzích ryb; koncentrace polycyklických aromatických uhlovodíků byla vyšší u plotic. Byly nalezeny bodové zdroje vstupu PCB jako hlavní třídy kontaminantů do prostředí. Koncentrace PCB dobře korelovaly s indukcí biomarkeru dioxinové aktivity – cytochrom P4501A-dependentní 7-etoxyresorufin-O-deetylázové (EROD) aktivity v jaterní mikrosomální frakci plotice i tlouště. Jaterní mikrosomální glutation-S-transferázová aktivita a cytosolová glutationreduktázová aktivita byly užity jako selektivní biomarkery oxidativního stresu. Modulace jejich aktivit environmentálním zatížením byly vzájemně velmi podobné; ovšem lišily se výrazně od profilu indukcí EROD aktivit a korespondovaly spíše s vyššími koncentracemi lindanu a DDT a jeho metabolitů než s obsahem PCB v odběrových místech.

biomarkery; PCB; organochlorové kontaminanty; PAH; CYP1A; dioxinová toxicita; oxidativní stres

INTRODUCTION

Concept of biochemical monitoring

The term "chemical monitoring" is used for repeated analytical measurements of concentrations of selected contaminant classes in abiotic and biotic samples. Biological monitoring is an assessment of exposure to or effects of environmental pollutants by determination of early adverse responses particularly on the biochemical, cellular and tissue levels. The biological monitoring is based on measurements of biomarkers defined as changes (biological responses) of the parameters under study related directly to the exposure to or toxic effects

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of environmental chemicals (WHO, 1993; Peakall and Walker, 1994).

Biochemical markers of toxicity are suggested to reflect specifically the interactions between the biological system and the hazardous chemicals, or effects of exposure, leading to such events as induction of detoxifying enzymes, production of adducts of xenobiotics with nucleic acids and other chemically altered cell components, and endocrine dysfunctions (Stegeman et al., 1992). Thus, they allow us to distinguish among various toxicity mechanisms and, in some cases, to quantify the impact of xenobiotics on the organism.

Analytical data are important particularly to the monitoring of accumulation of xenobiotics in animal and plant tissues and hence to the protection of the food chain. Biochemical monitoring is becoming another indispensable part of environmental risk assessment (Peakall and Walker, 1994; van Gestel and van Brummelen, 1996). Although the biochemical monitoring has a general importance, no set of the respective *in vivo* tests has yet been accepted and validated apart from biomarkers of what is called the dioxin-like toxicity. Currently, the efforts are oriented on the search for potential biomarkers including estimation of their specificity and sensitivity, development of tests of biochemical monitoring, and their application, validation and standardisation. The research consists in comparisons of analytical and biochemical data obtained in laboratory and field studies, search for specific bioindicators, and investigations of interrelations among the individual biochemical markers.

Biochemical markers of dioxin-like toxicity and oxidative stress in fish

A number of aromatic contaminants, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), coplanar and mono-*ortho*-chlorinated polychlorinated biphenyls (PCBs), some polycyclic aromatic hydrocarbons (PAHs), and further structurally related contaminants, induce a certain, fairly well described set of toxic effects (van den Berg et al., 1998). This dioxin-like toxicity mechanism and rate are specifically demonstrated by the induction of the hepatic cytochrome P4501A (Stegeman et al., 1992). Such induction can be used as a specific biochemical marker of exposure to contaminants of the dioxin type and/or of their toxic potential (Goksoyr and Förlin, 1992). The cytochrome P4501A is most often assayed as 7-ethoxyresorufin *O*-deethylase (EROD) activity.

Another significant toxicity mechanism that accompanies a number of pathologic effects, such as nongenotoxic carcinogenesis and promotion of carcinogenesis, is oxidative stress as a result of imbalance between prooxidative and antioxidative processes in organisms exposed to various xenobiotics (Winston and Di Giulio, 1991; Kelly et al., 1998). The most important of them are organochlorine compounds, PCBs, dioxins, and heavy metals (Pedrajas et al., 1995; Förlin et al., 1996; Otto and Moon, 1996; van der Oost et al., 1996).

Changes of enzyme levels and concentrations of low-molecular components of the antioxidative defence systems, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione, and vitamin E, were tested as possible biochemical markers of oxidative stress, but the results of laboratory and field investigations in several fish species were rather controversial (Winston and Di Giulio, 1991; Stegeman et al., 1992; Otto and Moon, 1995; van der Oost et al., 1996). On the other hand, prospective biochemical markers of increased oxyradical production include also hepatic cytosolic glutathione reductase. An induction of its activity was observed in model experiments in which rainbow trout were exposed to coplanar 3,3',4,4'-PCB (Otto and Moon, 1995), a commercial PCB mixture (Förlin et al., 1996), and p,p'-DDE or 2,3-dimethoxynaphthoquinone (Petřivalský et al., 1997). Using a method developed earlier, activation of hepatic microsomal glutathione-*S*-transferase (mGST) was demonstrated in rainbow trout treated with p,p'-DDE, or 2,3-dimethoxynaphthoquinone (Petřivalský et al., 1997) and in common carp contaminated with PCBs (Machala et al., 1997).

Only a few field investigations of *in vivo* biomarkers have been done in roach and chub as typical inhabitants of chemically more contaminated Central European rivers (Monod et al., 1988; Vindimian et al., 1991; van der Oost et al., 1994; Viganó et al., 1998). The objective of our investigations was to determine and compare basic and induced levels of biochemical markers of dioxin-like toxicity and oxidative stress in hepatic tissues of roach and chub caught at various sites of the Skalice river differing in the rate of chemical contamination.

MATERIAL AND METHODS

Chemicals

7-Ethoxyresorufin was purchased from Molecular Probes (Eugene, MI, USA), resorufin, NADPH, bicinchoninic acid, reduced and oxidised glutathione, 1-chloro-2,4-dinitrobenzene, and ethacrynic acid were purchased from Sigma-Aldrich (Prague, Czech Republic). PAHs, organochlorine and PCB congeners and isomers were obtained from Dr. Ehrenstorfer (Darmstadt, Germany). All other chemicals were of the highest commercially available purity.

Procedures

Adult 2- to 4-year-old roach (*Rutilus rutilus*) and chub (*Leuciscus cephalus*) were fished out using an electric aggregate at nine sites of the Skalice river in June 1995. Dorsal muscle and liver samples were collected for analytical determination of organic contami-

nants and measurement of biochemical parameters, respectively. The samples were frozen to –20 °C immediately after sampling.

Conventional procedures were used for the extraction, fractionation and analysis of PCB congeners, organochlorine pesticides and their metabolites, hexachlorobenzene, and PAHs. The latter were extracted with dichloromethane, separated by gel permeation chromatography, and determined by HPLC with fluorimetric detection using a NovaPak C18 column (Waters, Prague, Czech Republic). PCBs and other chlorinated contaminants were extracted with petroleum ether-acetone (50 : 50 v/v), separated by gel permeation chromatography, fractionated in a HYPERCARB column (50 x 4.6 mm, 7µm, Shandon, Runcorn, U.K.) and determined by HRGC/ECD on a DB-5 column (60 m x 0.25 mm, 0.25 µm, Quadrex Corporation, New Haven, NH, USA).

Individual liver samples were homogenised and the microsomal and cytosolic fractions were separated by differential centrifugation (Petřivalský et al., 1997) and stored at –80 °C until use. The EROD activity was measured fluorimetrically as described by Prough et al. (1978) using 2 µM ethoxyresorufin as the substrate. The activity of mGST was determined spectrophotometrically using 1-chloro-2,4-dinitrobenzene as the substrate for the conjugation reaction (Moorhouse and Cassida, 1992), and the activity of cytosolic glutathione reductase was also determined spectrophotometrically using oxidised glutathione and NADPH (Carlberg and Mannervik, 1985). All the enzyme activities were assayed at 30 °C. The concentration of proteins in the hepatic subcellular fractions was determined by the bicinchoninic method as described by Smith et al. (1985) using the BCA kit (Sigma Chem. Co., Prague, Czech Republic).

Statistical analyses were done using the STAT Plus software (Matoušková et al., 1992).

RESULTS AND DISCUSSION

Concentrations of major organic contaminant classes in fish muscles

Roach and chub were fished out at nine sites of the Skalice river (a tributary of the Otava river, South Bohemian region, Czech Republic). Descriptions of the sampling sites, the numbers of the collected samples and the results of analyses for organic contaminants in fish muscles, expressed as mg contaminant per 1 kg fat, are presented in Tab. I.

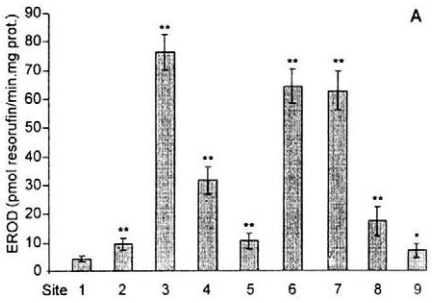
The highest concentrations of PCBs were found at sites 3 (downstream from a road metal coating plant), 6, and 7 (the town of Březnice). The concentrations of mono-*ortho*-chlorinated PCBs (Nos. 105, 118, 156) markedly predominated in the total PCB content in the samples collected at sites 3 and 6 (data not shown here).

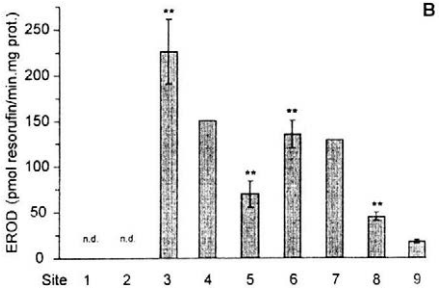
The sum of HCH in fish muscles corresponded almost exclusively to the concentration of lindane. The highest concentrations of HCH isomers were found at sites 4, 5 and 6. Higher concentrations of DDT and its metabolites were found almost throughout the sector of the river under study (sites 2 through 9). The concentrations of hexachlorobenzene were very low ranging from 2.15 to 26.9 µg per kg fat (data not shown here).

I. Concentrations of organic pollutants in muscle tissues of roach and chub collected in the Skalice river (mg/kg LW)

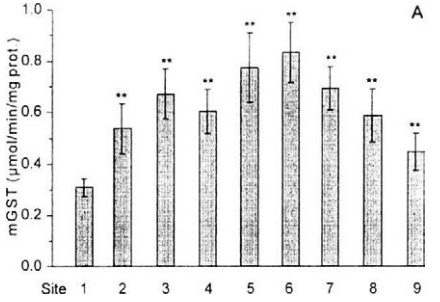
Site	Station	Species (sampling size)	Σ PCB[1]	Σ HCH	Σ DDT + DDE	Σ PAH[2]
1	Věšín	roach (n = 12)	1.08	0.21	0.96	0.54
2	Rožmitál	roach (n = 12)	4.15	0.25	3.11	0.81
3	Rožmitál 2	roach (n = 8)	18.12	0.37	4.27	0.51
		chub (n = 4)	14.10	0.22	3.19	0.22
4	Skuhrov	roach (n = 9)	11.66	1.32	2.90	1.52
		chub (n = 1)	n.d.	n.d.	n.d.	n.d.
5	Zadní Poříčí	roach (n = 8)	8.22	2.33	2.36	0.67
		chub (n = 9)	10.66	2.53	4.01	0.37
6	Březnice	roach (n = 8)	15.38	1.71	6.86	0.98
		chub (n = 5)	15.63	1.42	7.83	0.33
7	Březnice 2	roach (n = 2)	13.79	0.78	4.15	0.54
		chub (n = 1)	12.80	0.84	3.74	0.33
8	Myslín	roach (n = 7)	9.95	0.32	4.56	1.24
		chub (n = 3)	9.79	0.16	4.71	1.10
9	Nerestce	roach (n = 8)	n.d.	n.d.	n.d.	n.d.
		chub (n = 9)	8.11	0.20	4.64	0.70

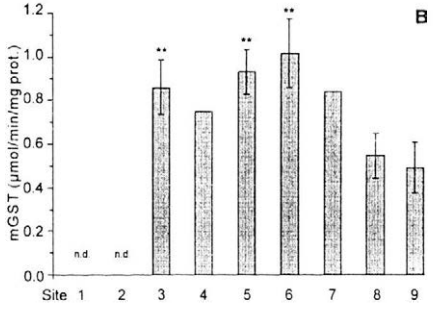
[1]sum of concentrations of PCB congeners, IUPAC Nos. 77, 126, 169, 105, 118, 156, 28, 52, 101, 138, 153, and 180
[2]sum of 16 non-alkylated polycyclic aromatic hydrocarbons
n.d. – not determined





1. Activities of cytochrome P4501A-dependent 7-ethoxyresorufin O-deethylase (EROD) in liver microsomes of roach (A) and chub (B) collected at nine sites of the Skalice river (for locations and sampling sizes see Tab. I); * $p < 0.05$, ** $p < 0.01$





2. Microsomal glutathione *S*-transferase (mGST) activities in the liver of roach (A) and chub (B) collected at nine sites of the Skalice river (for locations and sampling sizes see Tab. I); ** $p < 0.01$

PAHs rank with the less persistent and relatively readily metabolised xenobiotics and the respective analytical data are suggestive of a long-term exposure. The highest concentrations were found in roach and chub fished out at sites 4 and 8, respectively.

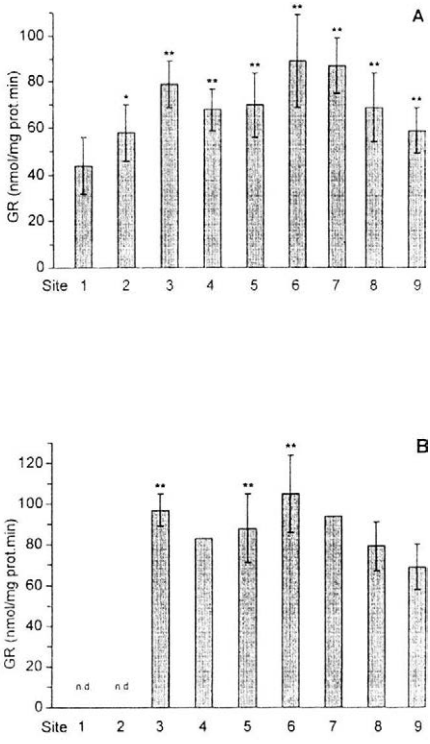
Except for higher concentrations of PAH in roach, no marked differences were observed between the two fish species in the accumulation of the contaminants under study in the muscular tissue.

Hepatic biochemical markers of dioxin-like toxicity and oxidative stress

The above analytical data were completed with values of specific ecotoxicological parameters (selected biochemical markers) showing the actual toxic impacts of the aquatic contaminants on the fish species under study.

The microsomal EROD activities, reflecting the overall effect of contaminants inducing the dioxin-like toxicity, were measured in hepatic tissue of roach and chub (Fig. 1) using those found in roach sampled at site 1 (Věšín) as the reference values for the roach. The highest induction of EROD was found at site 3 (17.7-fold as compared with the reference value) and at sites 6 and 7 (Březnice). The minimal rate of contamination with chemicals of the dioxin type was found at site 9 (Neresice) (Fig. 1A). Since no chub were fished at site 1, the activities found at this site were used as the reference value for this species. The highest EROD activity in the hepatic microsomal fraction of chub was found at site 3 (12-fold increase as compared with the reference value) and somewhat weaker responses were recorded at sites 6 and 7 (Fig. 1B).

A high induction rate of the EROD activity was reported previously in roach (Monod et al., 1988) and in chub (Vindimian et al., 1991). The present study has confirmed these findings. Although the inducibilities of the EROD activity in both fish species under study were similar, the levels were markedly higher in chub. The induction of the EROD activity correlated well with the concentration of PCBs including the mono-*ortho*-chlorinated congeners, although a possible minor contribution of PAHs at sites 4 and 8 should not be disregarded. The biochemical data indicated the existence of point sources of contamination with chemicals of the dioxin-like toxicity. Compared with the reference sampling site, an induction of the EROD activity in hepatic tissue was demonstrated at all the remaining sampling sites. It can therefore be concluded that this biomarker is highly sensitive indicating at least the presence of concentrations > 4 mg of PCBs per kg fat



3. Cytosolic glutathione reductase activities in the liver of roach (A) and chub (B) collected at nine sites of the Skalice river (for locations and sampling sizes see Tab. I); * $p < 0.05$, ** $p < 0.01$.

(calculated as the sum of 7 indicator PCB congeners). The objective of continuing studies is to define species-specific values of EROD induction indicating a significant manifestation of effects caused by TCDD and the related planar polyaromatic hydrocarbons.

An increase of prooxidative processes was evident from both the microsomal (membrane) GST (Fig. 2) and the cytosolic glutathione reductase (Fig. 3) activities. The highest activity of mGST was found in roach at sites 5 and 6 where also the highest concentrations of lindane (site 5) and DDT incl. its metabolites (site 6) were demonstrated (Tab. I). Moreover, a high activity of mGST was found in chub at site 3 (Fig. 2B). A similar pattern of induction was also found for glutathione reductase, although this enzyme was less inducible and the effects of further contaminants, such as PCB, were probably stronger. In terms of the activity, the order of the sampling sites was as follows: 6 > 7 > 3 (Fig. 3). Generally, the glutathione-dependent enzymes are less inducible than P4501A; in roach, the mGST activity at site 5 was increased to a 2.7-fold value and the glutathione reductase activity at site 6 to a two-fold value. The activities of the glutathione-dependent enzymes were moderately higher in chub than in roach.

Microsomal GST and cytosolic glutathione reductase belong to the complex cellular defence system against oxidative stress (Kappus, 1985; Petřivalský et al., 1997). An increase in hepatic mGST activity has been shown to reflect imbalances of prooxidative and antioxidative processes in the membranes of the endoplasmatic reticulum. Similarly, cytosolic glutathione reductase activity can be induced as a result of exposure to chemicals causing oxidative stress (Petřivalský et al., 1997; Kelly et al., 1998). The usefulness of determination of mGST and glutathione reductase activities in biochemical monitoring of oxidative stressors in fish has been demonstrated in the present study as well as in a few recent papers (Otto and Moon, 1996; Förlin et al., 1996; Machala et al., 1997). Lower biomarker responses to contaminants including the EROD and glutathione reductase activities, were reported in chub in comparison to other cyprinoids (Viganó et al., 1998). However, our results have shown unequivocally that this species exhibited a suitable inducibility of hepatic EROD and mGST and GR activities in areas with different contamination rates.

The biomarkers are considered the integral parameters of specific impact, dioxin-like toxicity or oxidative stress. The results presented here do not allow us to adjoin a specific class of contaminants determined by analytical methods to a specific biomarker of oxidative stress. Nevertheless, the pattern of EROD activities differed from that of activities of the glutathione-dependent enzymes. The manifestation of oxidative stress correlated with increased concentrations of lindane and DDT metabolites and less with increased concentrations of PCBs. The induction of the EROD activity has been accepted as a specific biochemical marker of the dioxin-like activity (Stegeman et al., 1992). The oxidative stress parameters are complementary data yielding information on other mechanism(s) of toxic effects of aquatic contaminants.

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The author shall give his full name (and the names of other collaborators), academic, scientific and pedagogic titles, full address of his workplace and postal code, telephone and fax number, or e-mail.

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