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Review Paper

DIAGNOSTIC METHODS OF CARDIOMYOPATHY IN DOGS - OLD AND NEW PERSPECTIVES AND METHODS

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Summary: Dilated cardiomyopathy (DCM) is an important cause of morbidity and mortality especially in large breed dogs. New diagnostic methods have been developed in the past few years to diagnose the disease early and improve chances of survival. Familial predisposition to DCM in some breeds opens possibilities of the early diagnosing by genetic testing and research has been done to find the causative molecular abnormalities. Doppler echocardiography and new sensitive methods like Tissue Doppler Imaging (TDI), a 24 to 48 hours electrocardiography (Holter monitoring), and cardiac biochemical markers such as atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and cardiac troponin I are –among others – the most promising methods in detection of DCM.

Key words: Doberman pinscher dogs; dilated cardiomyopathy; genetics; echocardiography;

Introduction

Cardiomyopathies are diseases of the myocardium associated with cardiac dysfunction. Dilated cardiomyopathy (DCM) is characterized by dilatation and impaired contraction of the left or both ventricles (1).

DCM occurs more frequently in large and giant breed dogs (for example Doberman pinscher, Irish wolfhounds, Saint Bernard dogs, German boxers, Great Danes, German shepherds, Newfoundlands and bull-mastiffs). A relatively high prevalence has been reported in medium sized dogs also (English and American cocker spaniels and Dalmatians) (2, 3, 4). In Slovenia, most affected breeds are: Doberman pinscher, German shepherd, German boxer, Great Dane, and Rottweiler (5).

The most common form of DCM in dogs is idiopathic DCM, because many of the processes that could lead to myocardial injury in dogs remain to be determined. Causes such as genetic factors, toxic factors, immunologic and viral aetiology have been theorised upon and remain to be proved (2).

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Most of the available data on the natural course of DCM development have been collected by observing Doberman pinscher dogs. DCM in Doberman pinscher dogs is a slowly progressive primary myocardial disease with a familial predisposition, which develops in two distinct stages: the first phase is a protracted (≥ 2 to 3 years long) occult phase characterised by evidence of echocardiographic and electrical deviations in the absence of clinical signs of heart disease. The echocardiographic abnormalities consist of left ventricular (LV) enlargement in systole and/or diastole that will progress to clinical phase with signs of left sided congestive heart failure (CHF). The electrical abnormality consists of the presence of ventricular premature contractions that can progress to more severe ventricular tachyarrhythmia, leading to sudden death. Approximately 30% of affected dogs die suddenly during the occult phase (2, 3). Screening is recommended for dogs with prevalence of DCM to identify dogs with cardiac abnormalities.

The diagnosis of overt DCM is not problematic, although it requires the exclusion of other cardiac, pulmonary or systemic etiologic factors of heart dilatation and hypokinesis. It is based on history and clinical findings (physical examination, ECG, radiographs, ultrasound) and other clinical tests

like haematology and biochemistry to exclude other primary or concurrent diseases (4).

The therapy and prognosis are determined in relation to the severity of CHF. The onset of clinical signs often means poor prognosis, but therapy usually prolongs life and alleviates symptoms. New studies are therefore oriented towards the early diagnosis of occult DCM.

This article reviews the latest development in the diagnostic methods of DCM in dogs to find out early changes that occur in the preclinical stage of the disease.

Echocardiography

Echocardiography is a non-invasive diagnostic method which provides valuable data concerning cardiac morphology and function. It is a highly operator-dependent diagnostic tool that relies on proper collection and interpretation of results.

Measurements of cardiac dimensions by observing chamber dimensions and wall thickness can reveal changes in geometry and dimensions because of the cardiac disease. Measurements of the left atrium (LA) and left ventricle (LV) give us insight of considerable dilation in diastole and systole. Indices of global LV systolic function, such as fractional shortening (FS) and ejection fraction (EF) can be calculated from the collected data.

The diagnosis of DCM is made on the basis of left ventricular dilatation, a lowered systolic function, and increased LV sphericity, where LV length has decreased. Breed specific echocardiographic data have been published for some breeds: Afghan hounds, beagles, German boxers, Cavalier King Charles spaniels, cocker spaniels (English), corgi (Pembroke), deerhounds, Doberman pinschers, golden retrievers, Great Danes, greyhounds, Irish wolfhounds, Newfoundlanders and miniature poodles (3)., In Doberman pinschers, left ventricular diastolic internal dimension (LVIDd)>45 mm in dogs weighing less or as much as 42 kg, and LVIDd>49 mm in dogs weighing over 42 kg, were proposed as abnormal by Calvert et al., who have followed these dogs for years (6). Similar observations were made by O'Grady, who suggests that, an LVID at end diastole of greater than or equal to 49 mm or at end systole of greater than or equal to 42 mm, is highly predictive for occult DCM in Doberman pinschers independent of the size of the dog (2). Despite this O'Grady believes that these criteria are not suitable for either small (especially female), or extremely large (especially male) Doberman pinschers. (2),

LV sphericity is measured so that LV diastolic length (in right parasternal two-dimensional long axis view) is divided by the M-mode diastolic value. An adapted scoring system based on major and minor criteria has been suggested for the identification of animals in the preclinical phase. Major criteria include the LV systolic and diastolic dimensions exceeding 95% confidence intervals of the normal M-mode values relative to breed, height and age, increased sphericity of the LV, FS of less than 20% to 25% (depending on breed specific values) and LVEF less than 40%. The proposed minor criteria include the presence of arrhythmia in predisposed breeds (e.g. Dobermans or boxers), atrial fibrillation, increased mitral valve M-mode E point to septal separation (EPSS), increased pre-ejection period and ejection period ratio (PEP:ET) over 95% confidence intervals, FS in equivocal ranges and left or bi-atrial enlargement (3). The two-dimensional display of left atrial diameter in right short-axis view at the level of the heart base exposes a wider portion of the left atrium and is commonly used measurement in comparison to aortic diameter.

The determination of cardiac function depends on exact measurements of cardiac dimensions. Improper alignment, poor image quality, and inadequate delineation of the endocardial blood pool interface represent technical difficulties that may yield erroneous results.

Fractional shortening (FS) is the major indicator of systolic function in veterinary echocardiography. Values of FS between 20% to 25% in Doberman pinschers are considered equivocal findings, but FS less than 15% suggests strong evidence of DCM, although values from 18% to 22% have been observed in normal Doberman pinschers (2). In humans, an ejection fraction (EF) is more important for the evaluation of the systolic function because FS may be deceptive in a concomitant severe mitral regurgitation. In their proposed guidelines for the diagnosis of DCM, Dukes – McEwan et al. consider less than 40% EF determined by 2D echocardiographic images (modified Simpson's rule) from the right parasternal long axis four chamber view as abnormally low. The end diastolic and end systolic ventricular volumes can be normalised to the body surface area (BSA) and expressed in ml/m^2 as end diastolic volume index (EDV-I) and end systolic volume index (ESV-I). ESV-I over 80 mls/m^2 is considered as elevated in regard to the reference <30 mls/m^2, which is suggested as normal canine ESV-I. The

authors have presumed that the diastolic dysfunction follows the systolic dysfunction. Some authors believe that the diastolic function can be altered and be an early sign of ongoing systolic dysfunction (7). The diastolic function can be assesed with mitral and pulmonary vein Doppler inflow velocity, tissue Doppler imaging of the mitral valve annulus velocity, and colour M-mode imaging of mitral valve opening velocity.

The evaluation of mitral inflow and PVF with pulsed wave Doppler to establish diastolic function is rather used as a prognostic tool than as a screening test. In human medicine, Doppler evaluation of transmitral flow (TMF) and pulmonary venous flow (PVF) is used to evaluate the diastolic filling activity. A restrictive filling pattern is usually present in patients with DCM and is related to a poor prognosis also in dogs, especially with constrained mitral inflow and a short E wave deceleration time (TDe)(<80 ms). The authors have not found any of the standard echocardiographic parameters (FS, mitral EPSS, ESV-I and EDV-I) to be related with survival rate (3).

In a recent study, O' Sullivan et al. investigated the diastolic function by means of combined echocardiographic indices (TMF, IVRT, PVF, Vp, and mitral annular motion) in normal Doberman pinschers and Doberman pinschers with occult and overt DCM. They considered left ventricular flow propagation velocity (Vp) with color mode echocardiography and mitral annular motion by TDI as load independent parameters unlike TMF, IVRT and PVF. They concluded that TMF, IVRT and PVF values vary according to the progression of diastolic dysfunction. TDI can be used to predict time to HF or SD in occult DCM (7).

One of the most significant uses of TDI is the detection of changes in myocardial function that cannot be displayed with conventional methods. It can be used as a complementary diagnostic method in patients with inconclusive echocardiographic results and it represents an important development in the preclinical diagnosis of cardiomyopathies (8).

Early diagnosing of asymptomatic myocardial abnormalities in dogs by TDI was confirmed by Chetboul et al. by dog model of Duchenne's cardiomyopathy (9, 10). Duchenne's muscular dystrophy is a hereditary muscular disease related to the formation of dysfunctional dystrophin, a structural protein of the myocyte. The dystrophinopathy causes not only the dysfunction of the skeletal muscles, but also myocardial alterations, which may be asymptomatic over a variable period of time. TDI parameters of regional myocardial function determined in a short axis view by applying M-mode TDI, were altered also when global ventricular function (left atrial and LV end-systolic and end-diastolic dimensions, LVFS%, wall thickening and E/A ratio), measured by conventional echocardiographic techniques, was still within normal limits. Systolic and diastolic myocardial velocity gradients (MVG) were lower in Golden Retriever muscular dystrophy (GRMD) dogs than in normal dogs, corresponding to a considerable reduction of systolic and diastolic endocardial velocities. Epicardial velocities were comparable in both groups. The ratio of early to late diastolic MVG was lower in GRMD dogs than in control dogs. According to these findings, systolic MVG was the most reliable parameter for distinguishing GRMD dogs from the control dogs (9).

Golden retriever muscular dystrophy (GMRD) is an inherited neuromuscular disease, similar to the Duchenne dystrophy in humans that eventually leads to GMRD associated DCM. These results in the preclinical phase of the GRMD associated cardiomyopathy confirmed TDI as a possible screening method of DCM (10, 11). Myocardial fibre strain is directly related to left ventricular (LV) contractility. Strain rate can be estimated as the spatial derivative of velocities (dV/ds – difference in velocity/difference in strain) obtained by tissue Doppler echocardiography (TDE). The use of TDI and strain rate imaging (SRI) as diagnostic tools of occult DCM and prognostic tools in dogs with overt DCM has to be evaluated. Recent studies focused on the determination of LVFW radial and longitudinal myocardial contraction velocities in normal dogs and in dogs with DCM revealing intramyocardial radial and longitudinal gradients (12). LVFW velocity gradients in dogs with idiopathic DCM decrease, and LV contractility along the short and the long axis is impaired in systole and in diastole (13).

Introducing new, preload independent parameters may enhance the evaluation of the LVFW diastolic function. Flow propagation velocity (Vp) from a colour M-mode recording and mitral annular motion by PW TDI are measurements that can be added to the Doppler echocardiography which combines transmitral flow (TMF), isovolumic relaxation time (IVRT), pulmonary venous flow (PVF) as preload dependent indices of diastolic function. Doberman pinscher dogs in occult and clinical DCM show altered TMF, reduced systolic to diastolic PVF ratio and reduced Vp. Shorter early TMF deceleration time (DTe) is correlated to the approaching onset of CHF or sudden death (7).

Dobutamine stress echography (DSE) is based on the fact, that increasing myocardial inotropy with dobutamine can reveal occult cardiac dysfunction, which is not evident when the animal is at rest. It can be safely performed as a non-invasive diagnostic method, complementary to conventional clinical examinations. The echocardiographic parameters (morphology and filling pattern) are measured by 2D guided M-mode echocardiography, and colour flow Doppler imaging (14, 15, 16). Minors and O' Grady evaluated echocardiographic parameters in healthy Doberman pinschers and in Doberman pinschers with occult DCM when animals were at rest and under the dobutamine stress. (14) As a result of the study, they were able to detect asymptomatic Doberman pinschers with an early cardiac dysfunction even when the left ventricular end-diastolic dimension and FS were within normal limits. Indices of systolic function when the animals were at rest (left ventricular dimension at end-systole: LVID-S, PEP, and PEP/left ventricular ejection time: LVET ratio) and under DSE (LVID-S, FS, wall stress index at end-systole – WSIS) were reduced early in the course of the disease. Diastolic function in affected dogs was also altered when the animals were at rest (prolonged IVRT) and under DSE (E/A ratio was significantly lower in dobutamine stressed occult DCM dogs than in healthy dogs) (14). New echocardiographic techniques and new software solutions can provide a lot of information regarding the functional status of the myocardium. Parameter values have to be determined to provide a reference for the operators, considering the influence of a breed, body weight, and age on the results. These parameters should be included in screening examination protocols for breeds predisposed to DCM.

Echocardiographic prognostic indicators for dogs with dilated cardiomyopahy have been suggested by Borgarelli et all. Severity of heart failure, ascites, end-systolic index greater than 140 mL/m^2, EF <25%, and restrictive transmitral flow,significantly affected the survival time period in a negative way. (17)

Electrocardiography

Electrocardiography has proved to be useful in boxers and Doberman pinscher dogs for the identification of dogs prone to developing DCM. As a single ECG recording corresponds to a small fraction of the dog's cardiac rhythm, a 24 to 48 hours ECG (Holter monitoring) is required to asses the heart rhythm over an extended time period (3, 4). The ambulatory electrocardiography is necessary to quantify heart rates and rhythms, and to identify abnormalities indicating cardiomyopathy. Especially in Doberman pinscher dogs, indications of possible onset of DCM include presence of ventricular premature contractions (VPC) and/or ventricular tachycardia (VT) (6, 18, 19, 20). Heart rate variability is reduced only in dogs with severe myocardial failure and can not provide additional information regarding the severity of LV dysfunction or risk of sudden death (20,21).

Most clinically healthy Doberman pinschers with normal ECG results have no or few VPC/24 h. Most Dobermans that are less than 4 years old have <1 VPC on an ambulatory recording. The number of dogs with detectable VPCs increases with age, but the number of VPC is <10 VPC/24 h. More than 50 VPC/24 h or ≥1 couplet or triplet of VPC/24 h, are predictive of development of DCM (19). In Doberman pinschers, the severity of arrhythmia tends to progress with the degree of myocardial failure, and sustained ventricular tachycardia persisting >30 seconds can be correlated with the risk of a sudden death, whereas less severe arrhythmias are not (3, 20).

Calvert et al. focused their studies in the electrocardiographic evaluation on clinically normal client-owned Doberman pinschers (mean age: 5 to 7 years) with abnormal or equivocal echocardiographic findings (6, 18). The echocardiographic observed were: LVIDd, LVIDs, LVFS, Mitral valve (MV) EPSS. The following values: LVIDd >50mm; LVIDs >40mm; LVFS ≤25%mm were considered abnormal, and LVIDd 48-50mm; LVIDs 38-40mm; LVFS 26-29% values were considered equivocal (18-20). In these studies, all the dogs with echocardiographic abnormalities (abnormal and equivocal) showed an elevated number of VPC during 24 Holter monitoring (6, 18). A follow-up study confirmed the correlation between the electrocardiographic abnormalities and the possibility to develop dilated cardiomyopathy (19).

The signal-averaged electrocardiography (SAECG) technique involves a computerized analysis of the standard electrocardiogram (ECG). This method allows the operator to detect small electrical impulses that are often covered by skeletal muscle activity and other extraneous sources of "noise" when recording a surface ECG. This low amplitude impulses that follow the QRS segment are defined as ventricular late potentials (VLP) (23). VLP result from regions of myocardial fibrosis. They are related to an increased risk of ventricular tachyarrhythmia and a sudden cardiac death, and occur in patients

with cardiac abnormalities, especially coronary artery disease, or following an acute myocardial infarction (23-26) and have been studied in patients with congenital muscular disorders (27). Calvert et al. in a study, conducted on Doberman pinschers with occult DCM, conclude that dogs with abnormal SAECG results are at higher risk of a sudden death than dogs with normal SAECG results. Despite this, the possibility of a sudden death cannot be excluded in patients with normal SAECG results (24).

Electrocardiography – in particular ambulatory ECG – is an indispensable tool in diagnosing occult DCM, especially in combination with an accurate echocardiographic examination. SAECG is a useful technique to assess the risk of sudden death, and as an index of myocardial dysfunction.

Biochemical markers of cardiac dysfunction

The usefulness of biochemical markers in the diagnosis and prognosis of heart disease is well documented in humans and there is an interest in identifying their relevance in companion animals. The prospect of identifying dogs (and cats) with asymptomatic heart disease via biochemical testing is exciting from several points of view. The introduction of new, simple and standardised laboratory methods for diagnosing heart structural changes would enable the individuals without extensive training in cardiology to identify animals with heart disease more reliably. It is not known how early in the course of a disease the changes can be identified because the data published to date include all animals with advanced disease. Biochemical testing might also help to clarify the status of dogs with equivocal results when evaluated by other diagnostic methods. Other advantages of biochemical testing are of practical nature: sample collection is minimally invasive and easy, the availability of tests is high, and these methods have become quantitative, repeatable, and economically affordable (28).

There are two types of biochemical markers. The first group comprises biochemical markers of myocardial injury and necrosis as cardiac enzymes creatine kinase (CK) and its myocardial fraction CK myocardial band (CKMB), aspartate aminotransferase (AAT), lactate dehidrogenase (LDH), and the recently evaluated structural myocardial proteins – troponins. The cardiac enzymes showed a limited ability in detecting myocardial injury due to low specificity and sensitivity, characteristics that are attained by troponins. Myosin light chain 1 (MLC-1) and heart type fatty acid binding proteins (H-FABP) are newly discovered biochemical markers that have been found in elevated quantities in human patients with myocite injury, but further investigation is needed to evaluate these markers (29-31). The second group of biochemical markers is used to assess the degree of cardiac dysfunction and is represented by the plasma neurohormones. The most commonly used indicators of neuroendocrine activation are plasma norepinephrine (NE), the atrial natriuretic peptide (ANP), the B-type peptide (BNP), peptides of the renin – angiotensin system (RAS) – vasopressin and aldosterone and plasma big endothelin-1 (32, 33).

The activation of the neuroendocrine system is well known in human patients with congestive HF (32). This finding has been confirmed in veterinary medicine also, and correlated positively with heat failure groups and left atrial size (38). The concentrations of neurohormones are related to the severity of the disease, and could be used in identification of various clinical stages of the disease, or in assessment of a suitable therapy (33, 34). O' Sullivan et al., in a study conducted on Doberman pinscher dogs, conclude that increasing norepinephrine concentrations (1.5 nM in male dogs and 5.8 nM in female dogs respectively, compared to 0.7 nM in normal male dogs and 1.0 nM in normal female dogs respectively) in the advanced phase of overt DCM are predictive of poor prognosis (35).

Endothelin is a 21-amino-acid peptide with a strong vasoconstricting activity, positive inotropy, chronotropy, stimulation of the RAS and sympathetic nervous system (SNS), and with mitogenic effects. It is released from the vascular endothelium and cardiac myocytes in response to cardiac damage in a precursor form (big ET-1) which is cleaved in its biologically active form by the catalytic action of endothelin converting enzyme (ECE) (35, 36). Normal Big ET-1 values range from 4.6 to 9.1 pg/mL averaging at 6.5 pg/mL in male dogs, and from 3.6 to 5.6 pg/mL averaging 4.5 pg/mL in female dogs. Occult DCM male dogs presented 6.9 to 12.5 pg/mL (mean 9.3 pg/mL), and female occult DCM 4.4 to 7.1 pg/mL (mean 5.6 pg/mL), respectively. In the previously mentioned study, O' Sullivan et al. measured Big ET-1 in Dobermans at different stages of DCM. Only the group of Dobermans with overt DCM had increased values of Big ET-1 (10.2 to 14.9 pg/mL – mean 12.3 pg/mL in male dogs, and 11.6 to 35.4 pg/mL –mean 20.2 pg/mL in female dogs), thus adding ET to the biochemical markers of degree of cardiac dysfunction and prognostic indicators (as norepinephrine) (35).

The assessment of natriuretic peptides as biochemical markers of cardiac dysfunction in asymptomatic patients is one of the most attractive methods (37). Natriuretic peptides are natural antagonists of the RAS system. They are released from the myocites in response to increased cardiac pressure due to compensatory mechanisms. There are two relevant types of natriuretic peptides related to the diagnosis of DCM: the atrial natriuretic peptide (ANP) and the brain natriuretic peptide (BNP) (28).

ANP is mainly released from atrial myocytes in response to the increase in right or left atrial pressure. On secretion, proANP is cleaved to the biologically active 28-amino-acid C-terminal ANP and the 98-amino-acid N-terminal (NT)-proANP. The two peptides are secreted into the circulation in equimolar quantities. NT-proANP has a longer half-life in circulation, it is more stable and more easily measured in laboratory (28, 29, 37, 39). ANP concentrations are known to increase in dogs with mitral regurgitation, heartworm disease, and congestive heart failure. ANP can be used to distinguish cardiac from non cardiac causes of dyspnea in patients with clinical disease (42), although it has been demonstrated that NP assays are not relevant in detection of clinically undetectable mitral valve disease (40). Normal serum ANP values are 0,269±0,013 nmol/L, values in occult DCM raise to 0,346±0,033 nmol/L (40).

BNP is secreted predominantly by left ventricular myocytes secondary to volume expansion or pressure overload. It is a 32-amino-acid peptide that shares structural and biological similarities to ANP. It is released into the blood as pro-hormone proBNP and it is then cleaved into Nt-proBNP and BNP. Nt-proBNP is more suitable for testing due to its longer half life and higher concentrations. BNP or NT-BNP has been proven to be the most reliable marker of early DCM among all the others (37 – 40) and new studies are oriented in the evaluation of NT-BNP utility to differentiate cardiac and respiratory causes of coughing or dyspnea in dogs (42, 43). Normal values of BNP are 6.51±0.66 pg/mL, whilst 14.35±1.6 pg/mL are indices of a developing heart disease (40). Natriuretic peptide concentrations have been proved to correlate with class of heart failure in dogs and their measurement may allow veterinarians to offer pet owners a more accurate long-term prognosis. Natriuretic peptide assays may also be useful in monitoring the efficacy of therapeutic intervention.

The cardiac troponin complex consists of three subunits (I, T in C), that regulate the excitation-contraction coupling of the sarcomeric proteins. CTnI is the inhibitory component that prevents interactions between actin and myosin until intracellular calcium is bound by cTnC. CTnI is normally bound to the actin filament via cTnT, but it detaches in response to sarcomeric injury and is released into the cytosol and extracellular space. Acute and/or chronic cardiac injury induces release of these subunits into the circulation (especially cTnI and cTnT), where their levels are proportional to the severity of myocardial damage. Markers such as cTnI and cTnT are more specific for myocardial damage than previously used enzymatic markers such as lactate dehidrogenase and creatine kinase (28, 40, 44).

Cardiac troponin I (TnI)is a better biomarker of the cardiac injury than cTnT due to its high sensitivity and specificity. The close homology of cardiac isoforms among mammalian species allows using immunoassays developed for humans for rapid and accurate measurements of canine cTnI concentrations (44). Despite this, cTnI is a marker of myocardial injury and its values increase in various cardiac and noncardiac diseases that cause myocardial damage thus limiting the use of cardiac troponin assays to detect myocardial damage and to evaluate its degree. CTnI values <0.07 ng/mL are considered normal, and values >0.07 ng/mL are considered pathologic (40, 44, 46). The use of cTnI as a biochemical marker of myocardial damage and the prognostic tool has been reported in a recent study conducted on female dogs with pyometra (46). According to the case report, high troponin concentrations were a marker of severe myocardial damage following suspected heatstroke (47). Compared with clinically normal dogs, boxers with arrhythmogenic right ventricular cardiomyopathy had a significant increase in serum cTnI concentration (0.142±0.05 ng/mL for boxers with ARVC, 0.079±0.03 ng/mL for control boxers, and 0.023±0.01 ng/mL for control non-boxers) (48). CTnI is used in human medicine to diagnose the acute myocardial ischemia. Even though several studies regard the use of cTnI in veterinary medicine, the diagnostic use is still limited to a potential use (28).

The idea of a simple and routine assessment of cardiac dysfunction is becoming reality thanks to the biochemical markers of cardiac dysfunction. There many variables that changes during a heart disease. In veterinary medicine, however, the natriuretic peptides and cTnI are most promising and useful. New investigation is required to establish the reference values for the biochemical markers

mentioned herein, especially considering physical characteristics (body weight, gender, age).

Genetics

Another interesting field that has been developing rapidly to prevent DCM is based on genetic predisposition and patterns of inheritance. In dogs, familial DCM has been observed in a number of breeds, and in most of them an autosomal dominant mode of inheritance is suspected (50). Boxers and Doberman pinschers are breeds with a certified familial predisposition to DCM (51). A recent study by Meurs et al. proved that DCM in Doberman pinscher dogs is a familial disease with an autosomal dominant mode of inheritance, but further investigation is required to identify the causative gene (52). The latest studies have focused on the evaluation of candidate genes for DCM (53-56). The discovery of the causative genes would enable the practitioners to eliminate the carriers from breeding programs as a definitive diagnostic test for early detection of DCM.

Post mortem histological characterisation of DCM

A histopathologic evaluation of canine DCM established two histologically distinct types of idiopathic canine DCM: the »fatty infiltration-degenerative« type in boxers and Doberman pinscher dogs, and the »attenuated wavy fiber « type of DCM in many giant, large and medium sized dogs, including some boxers and Doberman pinschers (57, 58). A post mortem histopathological evaluation of the heart should be performed in all predisposed dogs to confirm DCM, or to discover changes that were not clinically manifested in order to discover latent carriers of DCM, and to exclude them eventually from the breeding programmes.

Conclusion

Clinically detectable DCM is not difficult to diagnose due to the typical signs of congestive HF, but usually this disease has a poor prognosis. In Doberman pinscher dogs, there is an occult stage of DCM that can be diagnosed in order to determine an early therapy for the affected dog to prevent the onset of HF. A similar pre – clinical phase can be detected in Boxers (59).

New echocardiographic methods, Holter monitoring and the biochemical markers of cardiac dysfunction are to date the most promising methods, not only in the early detection of DCM, but also for the evaluation of the severity of the disease and to estimate the response of the organism to the therapy.

Echocardiographic parameters can be used to detect morphological abnormalities, Holter monitoring can help quantify heart rates and rhythms and to identify abnormalities indicating cardiomyopathy, biochemical markers can provide information about the level of disease exposure, extent of injury, and prognosis.

There are studies that certify the ability of new diagnostic methods for occult DCM, but there are different results among operators, there are different criteria and different conditions. There is still a need to establish reference values, to standardize these methods and make them objective instruments for detecting and evaluating occult DCM. These parameters should be included in screening protocols for predisposed breeds.

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DIAGNOSTIČNE METODE ZA UGOTAVLJANJE DILATACIJSKE KARDIOMIOPATIJE PRI PSIH – PREGLED STARIH IN NOVIH POGLEDOV IN METOD

A. Domanjko Petrič, K. Tomsič

Povzetek: Dilatacijska kardiomiopatija (DKM) je pogost vzrok za obolevnost in smrtnost predvsem psov velikih pasem. Številne nove diagnostične metode so bile razvite v zadnjih letih z namenom čim prejšnje diagnoze bolezni in podaljšanja dobe preživetja. Družinska podvrženost DKM pri nekaterih pasmah odpira nove možnosti za zgodnejše odkrivanje bolezni s pomočjo genetskih testov in veliko študij je usmerjenih v iskanje molekularnih nepravilnosti, ki so odgovorne za njen nastanek. Doplerska ehokardiografija in nove občutljive metode, kot so tkivna doplerska ehokardiogrfija (TDI), 24- do 48-urno elektrokardiografsko snemanje (Holter monitoriranje) in biokemični markerji srca, kot so atrijski natriuretični peptid (ANP), možganski natriuretični peptid (BNP) in srčni troponin I, so najbolj obetajoče metode za zgodnje ugotavljanje DKM.

Ključne besede: dobermani; dilatacijska kardiomiopatija; genetika; ultrazvok; holter monitoring; biokmarkerji.

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Original Research Paper

HUMORAL IMMUNE RESPONSE TO BACTERIA *BORRELIA BURGDORFERI* S.L. IN DAIRY CATTLE AFTER EXPERIMENTAL EXPOSURE

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Summary: To acquire insights into the humoral immunal response (HIR) of grazing diary cattle naturally infected with *B. burgdorferi* sensu lato (s.l.), an experimental group of 3 adult dairy cows was artificially infected with the bacteria. Three cows were intradermally (i.d.) infected with living *B. garinii*, and another was immunized with dead bacteria in Freund's incomplete adjuvant. The taxonomic identity and clonality of the isolates were confirmed by PCR amplification of the intergenic spacer between the 5S and 23S rRNA genes (1). The immune responses of the polyclonal, IgM and IgG class of antibodies were monitored both with whole-cell sonicated IgG and IgM and polyclonal ELISA and with indirect immunofluorescent assays (IIF). The immune response of the three i.d. infected animals remained low, but was detectable and characteristic in course, whereas in the vaccinated animal the immune response was strong.

Key words: *Borrelia burgdorferi*; dairy cattle; serological diagnosis.

Introduction

Lyme borreliosis (LB) – the most common arthropod-borne infection in Europe (2, 3) and in the United States (4) is a complex multisystem disorder in humans and animals, caused by *Borrelia burgdorferi* sensu lato, a genetically diverse species complex of spirochetes, comprising at least nine genospecies (5, 6, 7, 8). The principal vectors of these spirochetes are ticks belonging to the genus Ixodes (5). The different genospecies of *B. burgdorferi* s.l, appear to cause distinct clinical manifestations of Lyme borreliosis in humans (9). Serological tests and other diagnostic procedures are normally conducted to confirm *B. burgdorferi* s.l. infections.

Immune responses to *B. burgdorferi* s.l. have been analysed in rodents (10, 11, 12, 13), game birds (14), dogs (15, 16), horses (17, 18, 19), cattle (17, 20, 21, 24, 25) and rhesus monkeys (24). In our experiment, a fresh tick-borne isolate of *B. garinii* with a low spirochaete count was used and introduced by intradermal inoculation. In humans, serological confirmation of the diagnosis has been complicated by the delay in the development of the humoral immune response (HIR) to the spirochaete (27, 28) and it has been shown that up to 10 to 12 % of clinically ill patients have no HIR at all (29). The low sensitivity of serological tests in man early in the infection course has been a particular problem (29, 30, 32). However, in animals infected with the bacteria, the clinical aspects are often unclear and serological test results are relied upon to determine the cause of the illness. As established in previous investigations, levels of specific polyclonal antiborrelial antibodies rise in the grazing cattle according to the number of infections or the age of the animal (17, 33). In humans, however, a study of 15 patients in the first weeks of illness, when erythema migrans or meningitis was present, revealed that 14 of them had weak to strong IgM response, and of those 6 had weak to moderate IgG response (34). In the past, indirect-fluorescent antibody (IIF) procedures were used to detect antibodies to *B. burgdorferi* (34, 35); later ELISA (36, 37), which can quantify the level of immunoglobulins and specific polyclonal antibodies, was introduced and replaced the time consuming IF method.

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Assessment of IgM, IgG and specific polyclonal antibody responses may be helpful in determining the disease status or response to therapy (23, 38, 39, 40).

Consequently in our study we investigated whether the immunological response after experimental infection of cattle exists, what is it's dynamic during the first 20 weeks and whether it is high enough to be measured using ELISA and IIF.

Materials and methods

Four Simmental cows, aged as follows: animal A 26 months, animal B 36 months, animal C 28 months and animal D 37 months, were selected for the trial. These were non-grazing animals which, after clinical, biochemical and haematological examination, were declared to be healthy; all were stabled from birth in the stall where only saw-dust was used for litter, and to the best of our knowledge had no contact with *B. burgdorferi* s.l. A negative serological reaction in the IIF test and ELISA confirmed this. Three animals (A, B and C) were intradermally inoculated with a pure culture of *B. garinii*, isolated and studied in Slovenia (1) in the subaxillar region. Animal D was immunized with dead *B. garinii* bacterias in Freund's incomplete adjuvant (Difco, Detroit, USA), and the procedure repeated 6 weeks later (booster). After taking blood samples for haematological, biochemical and serological investigations, and checking the general health of the animals, animals were infected as follows:

animal A, left subaxillar region, 5 times i.d., injections of 2 x 10^3 microorganisms in 100 µl of broth BSKII, right subaxillar region, single i.d. injection of 104 microorganisms in 100 µl of broth BSKII;

animal B, left subaxillar region, single i.d. injection of 104 microorganisms in 100 µl of broth BSKII, right subaxillar region, single i.d. injection of 104 microorganisms in 100 µl of broth BSKII;

animal C, left subaxillar region, 5 times i.d. injections of 2 x 10^4 microorganisms in 100 µl of broth BSKII, right subaxillar region, in doses of 5 times i.d. injections of 2 x 10^3 microorganisms in 100 µl of broth BSKII;

animal D, i.m., in the right *m. gluteus*, 10^9 microorganisms killed in 10 ml of Freund's incomplete adjuvant; the procedure was repeated after 6 weeks.

A tuberculin syringe set was used for injecting the bacteria. After infecting the animals, the remaining bacteria were monitored, and their viability evaluated. Blood was taken from *V. jugularis* and *V. epigastrica cranialis superficialis* with a 10 ml syringe, complete with siliconized wall (Sherwood-Monoject, Gosport, UK). After being centrifuged three times, 1 ml of each serum was frozen at -20 °C.

The blood samples were taken once a week over a twenty week period. During the experiment, a total of 84 samples were taken and analysed.

IIF

The antigen used in the test was a suspension of tick isolated *B. garinii*. After 20 minutes of centrifugation at 11,000 x g the culture was resuspended in PBS with 5 mM MgCl2 with a pH of 7.4. This suspension was centrifuged and the procedure repeated.

The sediment was resuspended in a PBS buffer with $MgCl_2$ and attenuated until 20 to 30 bacteria could be seen in the covered droplet at a magnification of 400 times. 12 µl of this suspension was poured onto object glasses (Bio-Merieux, Marcy l'Etoile, France), after being degreased for two hours in a suspension of equal parts of ethanol and acetone and then heated, three times in the flame of a gas burner. The object glasses were then dried for 12 hours overnight at 37° C, later put into methanol for 10 minutes, then wrapped in aluminium foil and stored at –70° C. The sera which were stored at -20° C were diluted 1 : 100 and 1 : 250. The positive control sera were taken from cattle immunized with the dead *B. burgdorferi* bacteria. The negative control sera were taken from a 3-month-old calf that was reared in a stable and which repeatedly tested negative in control tests. Labelling was performed with a rabbit anti-bovine gamma-globulin conjugated with FITC (DAKO, Glostrup, Denmark). The remaining procedures and methodologies have been preformed as described before (39).

ELISA

Bacteria *B. garinii* was used to prepare the antigen. After repeated centrifugation and washing of the culture in the PBS at 4° C, the microorganisms were disintegrated using ultrasonic disintegrator (8 x 15 seconds by 6000 Hz). After 30 minutes of centrifugation at 25,000 g, the protein content was checked in the supernatant from which proteins were concentrated to 1 µg/ml in an ultracentrifuge (Centriprep, Amicon, USA). The dilution of an antigen, 1:5000 in carbonated-bicarbonated buffered saline with a pH of 9.6 (Sigma, St. Louis, USA) and in a concentration of 5 µg/ml, was distributed across microtitre plates (NUNC, Roskfielde, Denmark) in quantities of 100 µg per well. After 18 to 20 hours of

binding, the wells were washed with PBS containing 0.05 Tween 20; (PBST; Sigma, St. Louis, USA). Then over the course of one hour, uncoated parts of the wells were covered with 200 µl PBST containing 1 % powdered milk (Merck, Darmstadt, Germany), pH 7.2 at room temperature. After washing four times with PBS-T-BSA, the plates were placed on a shaker and incubated for one hour at room temperature with PBS-T-BSA and in several dilutions of cattle sera (1: 500; 1 : 1000; 1 : 2000). After washing the plates four times, both the protein G-HRP (DAKO) which was used to identify antibodies, and the conjugate – goat anti-bovine IgG conjugated-HRP or IgM conjugated-HRP (DAKO) were added to the microtitre wells in quantities of 100 µl per well in a dilution of 1:1000. The remaining procedures were performed following the manufacturer's instructions for goat anti-bovine IgG conjugated-HRP or IgM conjugated-HRP, DAKO). For negative control, the negative cattle sera were used in a dilution 1 : 500 and for positive control the immunised cattle sera with a titre of antibodies 1 : 6400, which was confirmed in an IFA in dilution of 1 : 5000 in the well.

The quotient of determination (R2), received by means of correlation analysis, was an illustration measure (connecting curve in figures) of the general tendency of dynamics, together with a cubic function with the common equation:

$$Yreg.= b_0 + b_1t + b_2t^2 + b_3t^3$$

Results

The humoral immune response in the treated animals in the class of polyclonal antibodies was low. An improvement was recorded at the end of the second week, post infection (animals A, B, C and D) and peaks were observed after eight weeks animals B and C), and after the eleventh, nineteenth and twentieth weeks animals A and C), and in an immunized animal, after the fifteenth week (animal D). The highest average post infection values can be expected at around fifteen weeks (Fig 1).

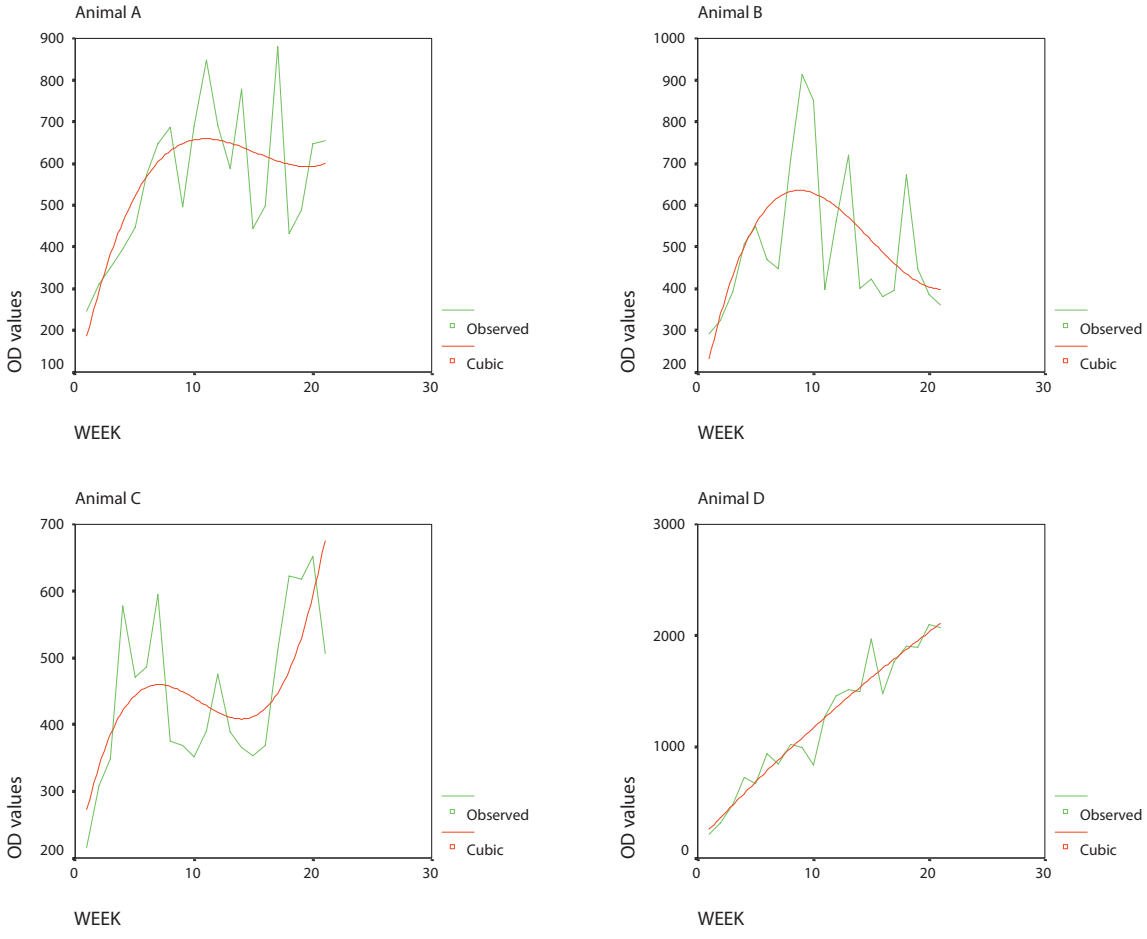

Figure 1: Levels of polyclonal antibodies response in treated animals measured by ELISA

Two weeks after infection, all animals showed a rise in the level of IgG. The highest values were recorded at the seventh and eighth weeks in animals A, B C and D and the fifteenth and eighteenth weeks (animals C and D). The highest values can be expected around 10.5 weeks (Fig. 2).

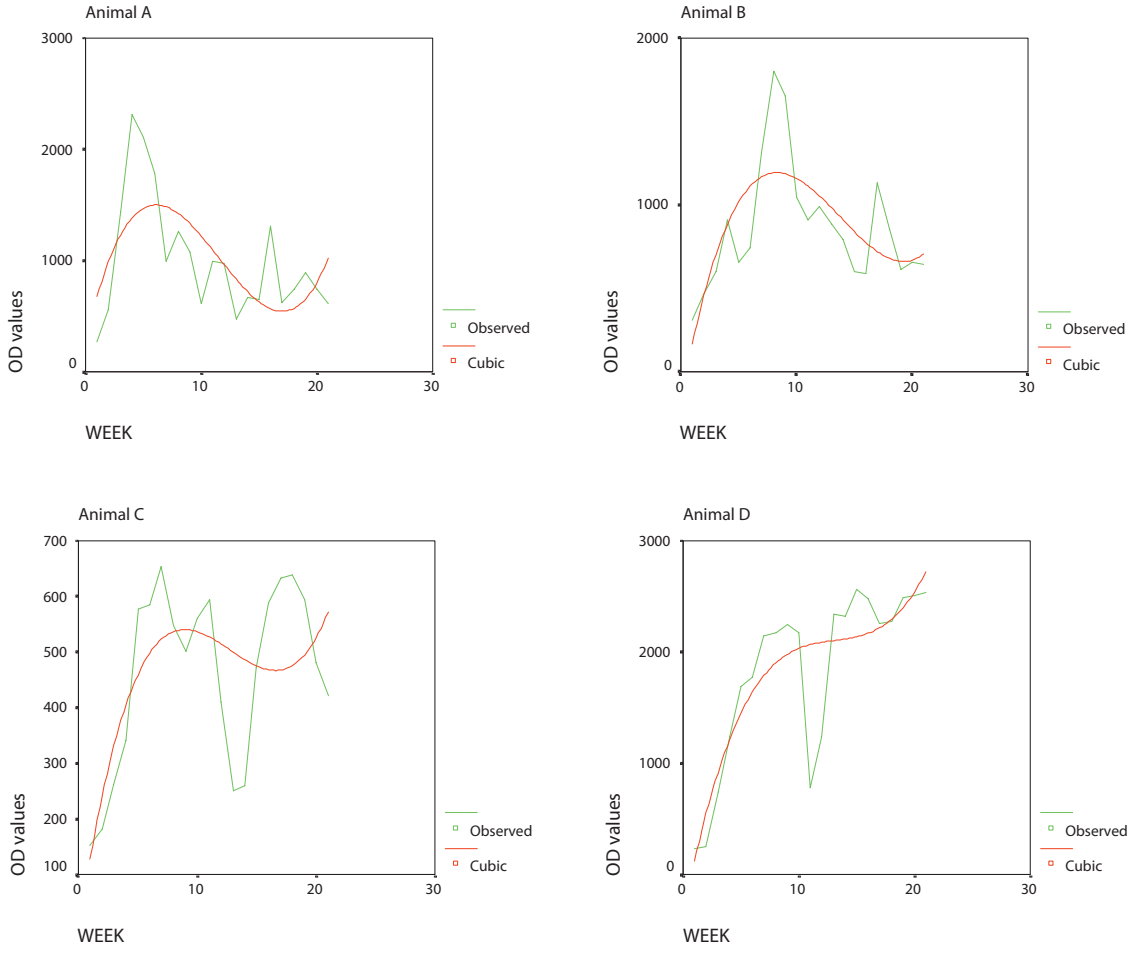

Figure 2: Levels of IgG antibodies response in treated animals measured by ELISA

The measurement of the IgM antibodies (Fig. 3) in animal A showed a rapid increase after the second week and reached the peak between the third and fourth week, in animals B and C between the eighth and ninth week, while in the vaccinated animal (animal D), the readings rose steadily until after the eighth week, and then declined, before again rising sharply between weeks twelve and fifteen. The highest values can be expected between the fourth and fifth weeks.

By comparing the values established by ELISA and IIF tests, it was concluded that there is a direct correlation between the two tests, with readings greater than 0.700 (OD values at 450/630) on the ELISA, comparable to readings of 1 : 256 and greater in the IIF test. This was considered highly reactive (+++ and ++). The range of 0.300 to 0.700 on the ELISA OD scale was selected as being definitely reactive (+). This was the equivalent to the range 1 : 64 to 1 : 256 in the IIF test. Optical readings, two standard deviations (SD) or more from the mean to 0.300 were interpreted as negative. The IIF test results matched (Fig. 4) the immune response values recorded by means of ELISA. Measurable, positive reactions were shown by the IIF test (dilution 1: 100) to start in weeks 2 (animal A, C and D) to 5 (animal B) in all animals.

The humoral immune responses resulting from experimental infection with *B. garinii* were weak, but detectable in animals A, B and C. However, a strong reaction was detected in the immunized animal (D), in which the value of the specific antibod-

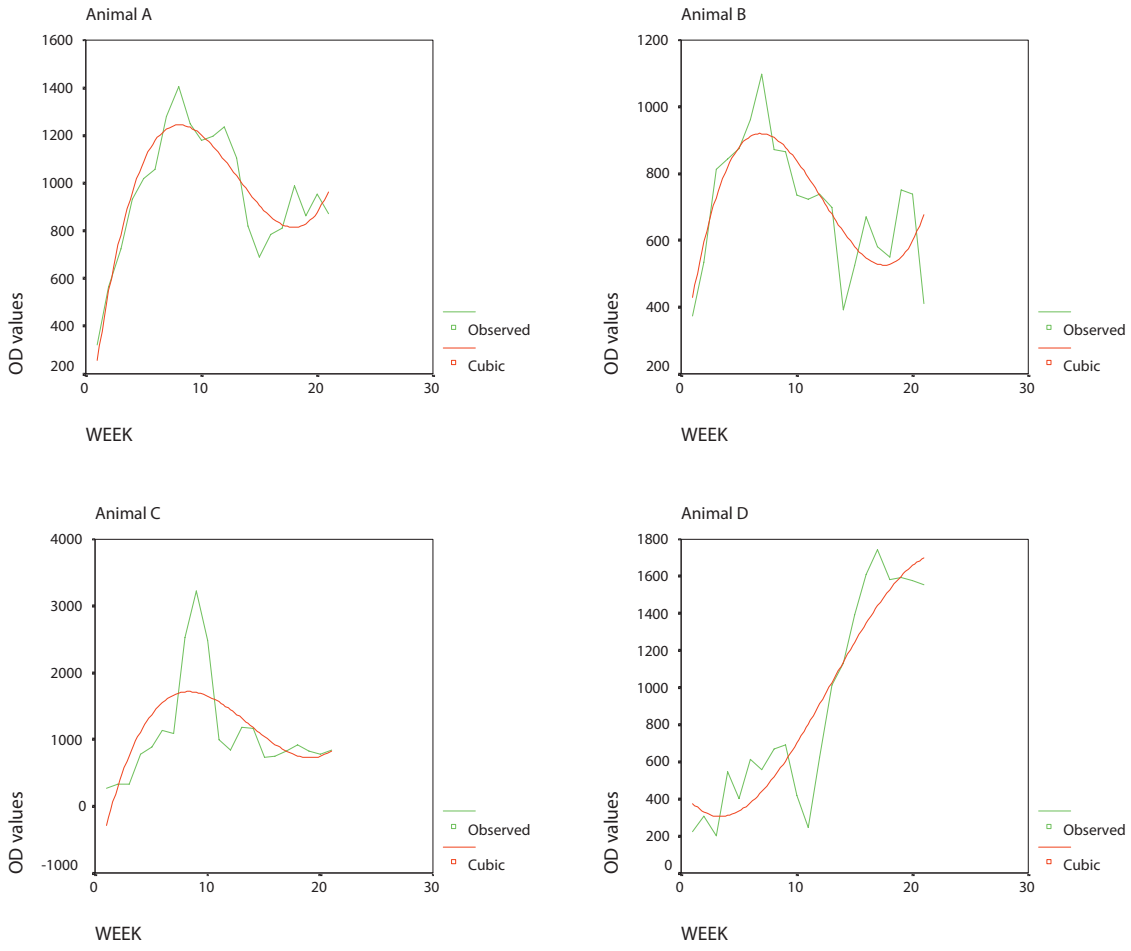

Figure 3: Values of IgM antibodies response in treated animals measured by ELISA

ies after 20 weeks showed a positive reaction in the IIF tests (dilution 1:12800) and exceeded 2680 units (by OD values at 450/630) or titer 1 : 38400 in the ELISA tests.

Discussion

The knowledge about the serological responses to Lyme borreliosis in animals, and especially in cattle, is limited and we are as yet unable to explain the numerous clinical events that could be connected to *B. burgdorferi* s.l. infection (swelling of the joints, lameness, interdigital dermatitis, emaciation).

In Slovenia, Lyme borreliosis is endemic, and is the most frequently diagnosed tick-borne disease in humans (40). Investigations carried out by the Veterinary Faculty in Ljubljana show that titres greater than 1:100 in IF seropositivity were detected in 18 % dogs, 24 % in wild animals (41) and 64 % in grazing cattle (32).

While the HIR in cases of Lyme disease generally follows a basic immunological pattern, it is common to see a delayed response, a quick elevation of IgG antibodies and in humans a complete absence of HIR (42), although less frequent (up to 12 %).

A rapid elevation of IgG was noted in all experimental animals and this is characteristic of secondary immune response. The same was observed in mice (43).

Specific and quite strong IgM response was detected in all four experimental animals.

In humans, the IgG response at the early stage of the disease is generally considered to be of little diagnostic value (45). Most patients have a good IgG response in the early stages of the disease and some reinfected patients may only have an IgG response (28). Our results suggest that the IgG-ELISA response can be of value in the serodiagnosis of early Lyme disease in the cattle. It is interesting to note that almost immediately after the second im-

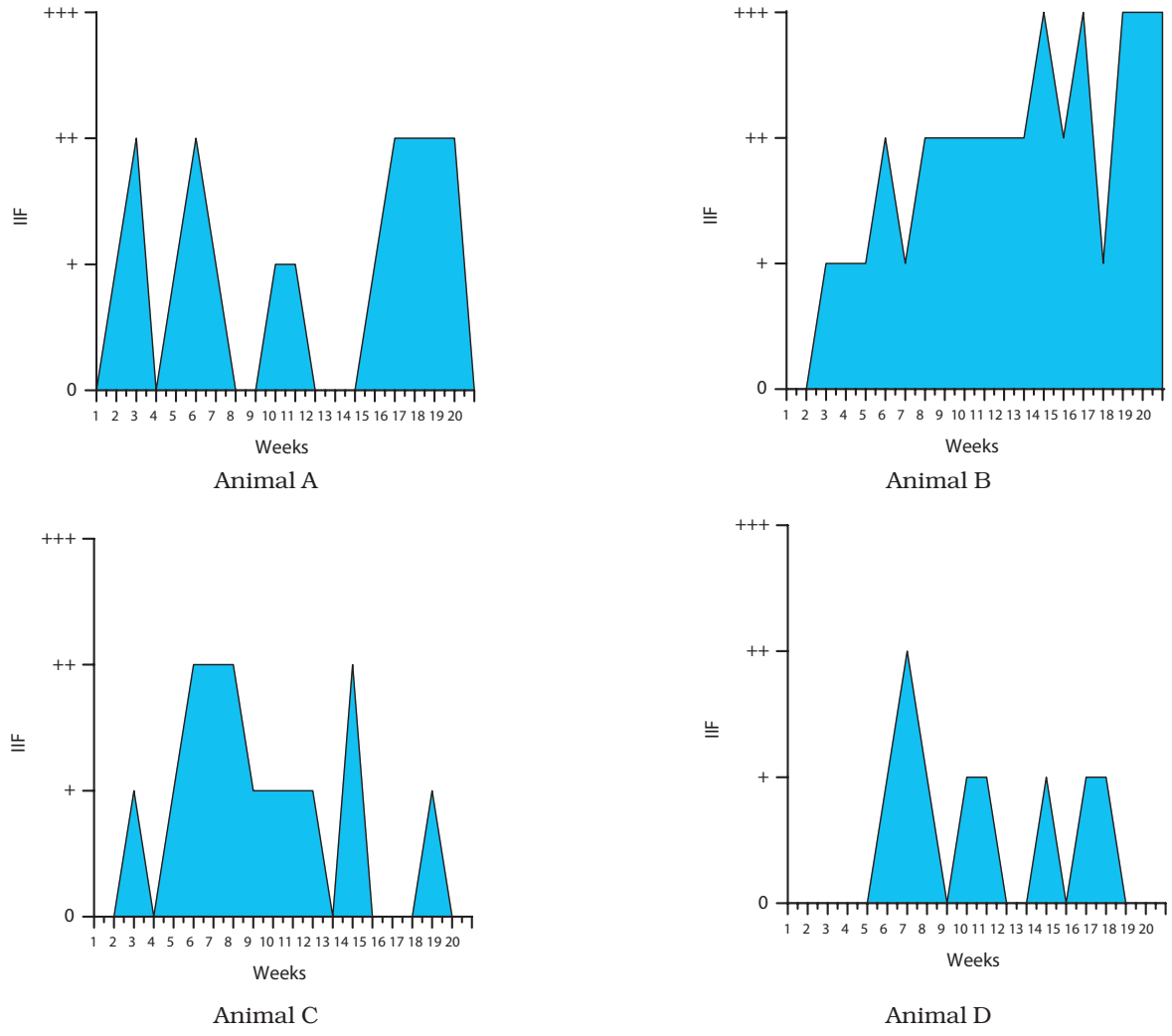

Figure 4: Specific polyclonal antibodies response in treated animals measured by IIF

munization (booster) in week 6, animal D displayed a quick fall in the levels of IgG and IgM antibodies. This continued until approximately the tenth week, before rising again. Although the predominant human isolate in Slovenia is the *B. afzelii* species (45), for our experiment we selected the tick-borne isolate of *B. garinii*, the first most frequently isolated species in animals and the second most frequently isolated species in humans in Slovenia(1, 46).

Multiple species of *B. burgdorferi* sensu lato differ in their molecular and antigenic properties, and therefore it could be expected that the recognition of a *B. burgdorferi* s.l. infection is difficult and dependent on the validity and specificity of the diagnostic assays and heterologous antigens used (47, 48).

It is important to note that the established specific early immune responses in our experiment may appeared due to homologous antigens of the same genospecies that were used in the tests. The same results were reported in the Finnish experiment, where only the homologous antigen (an antigen of the same genospecies) was sufficiently sensitive to give a positive result in the serological test (22). However, it is still not clear, what is the meaning of genospecies-binding differences of B. burgdorferi s.l. in pathogenecity and clinical manifestation of the disease in animals.

As established in this and other studies, the levels of specific anti-borrelial antibodies in grazing cattle depend on the number of infections or the age of animal (32, 35). The same pattern can be observed in humans, where HIR develops gradually over a period of months to years (28, 29, 30).

The weak immunological response established in our experiment probably mirrors the existing data, although the 20 - week period is not long enough to confirm this.

The results of serological investigations of clinically and subclinically ill animals are still not comparable to those in humans. In our previous study, it was established that basic HIR in grazing cattle in Slovenia is high, but the differences between the reactions of infected, though healthy animals, and those suspected of having Lyme disease, are not significant enough to be used as confirmation of the disease (32).

The enzyme-linked immunosorbent assay (ELISA) has been widely used for detecting antibodies to *B. burgdorferi* s.l. in human and veterinary medicine, and its efficiency and reliability is comparable with other available tests for Lyme disease (21, 44). These assays are still not standardised, thus requiring tests with various levels of sensitivity and specificity. As our results have shown, IIF and ELISA tests can be sufficiently sensitive to detect an infection in the cattle during the early phases of Lyme disease, but due to their weak humoral immunological response it is not easy to evaluate or use them. Generally, by comparing the values established by means of ELISA and IIF in our study, it was concluded that the IIF test results matched those of the immunological response values recorded by means of ELISA. Reactions in IIF tests in dilutions 1 : 100 were observed from 2.5 to 3 weeks (17 to 21 days) after infection.

It has been shown in several previous studies that humoral immunological response definitely indicates a reaction to *Borrelia burgdorferi* infected organisms; however since the strength of the HIR frequently depends on the phase of the illness, the clinical importance of HIR as a means of diagnosis is not yet clear.

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HUMORALNI IMUNSKI ODZIV GOVEDA PO POSKUSNEM STIKU Z BAKTERIJO *BORRELIA BURGDORFERI* S.L

Č. Borko

Povzetek: Da bi ugotovili značilnosti humoralnega imunskega odgovora (HIR) pašnega goveda, naravno okuženega z baketrijo *B. burgdorferi* sensu lato (s.l.), smo skupino treh odraslih goved umetno okužili z bakterijo. Tri goveda smo intradermalno (i.d.) okužili z živimi bakterijami *B. garinii*, četrto pa smo imunizirali z mrtvimi bakterijami v Freundovem nepopolnem adjuvansu. Taksonomsko identiteto in klonsko pripadnost izolata smo potrdili s PCR pomnoževanjem 5S in 23S r RNA (1). Ugotavljali smo imunski odgovor poliklonalnih, IgM in IgG protiteles z ELISO in z indirektno imunofluorescenčno analizo (IIF). Imunski odgovor treh intradermalno okuženih živali je bil nizek, vendar ugotovljiv in značilen v toku, medtem ko je bil pri vakcinirani živali visok.

Ključne besede: *Borrelia burgdorferi*; govedo; serološka diagnostika

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Original Research Paper

PROFILE OF STEROID HORMONES DURING OESTRUS AND EARLY PREGNANCY IN ARABIAN MARES

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Summary: Faecal and plasma steroid evaluations are well established approaches for monitoring reproductive function in mares. The purpose of this study was to detect the ovarian and uterine changes by transrectal ultrasonographic scanning, beside the estimation of gestagen and estradiol-17ß profiles in plasma and faecal samples of Arabian mares. Eight cyclic barren mares of different parities were used in the current work, and hormones were assayed using radioimmunoassay. The follicular growth was accompanied by a significant (P<0.05) decrease and increase in the profile of plasma progesterone (P4) and estradiol-17ß (E1-17ß), respectively. In addition, the minimum level of P4, and the maximum level of E1-17ß were detected at day 0 of ovulation. Similarly, the faecal progesterone metabolites (20∝-hydroxy-progesterone; i.e. 20∝-G) content showed a significant (P<0.05) decrease in its value starting from day -7 reaching its minimum level at second day post ovulation, meanwhile, the faecal E1-17ß content was reaching its maximum value on day 1 after ovulation. Following ovulation, the plasma P4 and faecal 20∝-G content showed a continuous significant (P<0.05) increase starting from the 3rd day post ovulation, and the levels of both faecal and plasma E1-17ß showed a continuous significant decrease. The levels of P4 in plasma and 20∝-G in faeces increased significantly (P<0.05) at day 14 to day 45 of gestation than those recorded at day 0 in nonpregnant mares. Moreover, the levels of E1-17ß in plasma and faeces increased significantly (P<0.05) at days 21 up to 45 of gestation than those estimated during 14th day of gestation as well as in nonpregnant mares. In conclusion, both ultrasonography and analysis of P4 and E1-17ß in plasma, and 20∝-G and E1-17ß in faeces have a predictive value for assessment of the follicular sizes, ovulation time and early pregnancy in Arabian mares.

Key words: arabian mare; estradiol-17ß; gestagens; ultrasonography; oestrus; gestation

Introduction

Determination of the reproductive status is one of the most important factors for effective management and efforts to use assisted reproductive techniques depend on the knowledge of the basic reproductive physiology of a given species (1) Several studies had been made to determine ovulation time in mares including the clinical and ultrasonographical examinations (2, 3, 4, 5). Ovulation was also predicated in oestrus mares by serial measurements of peripheral estrogen and progesterone concentrations (6). The maximum diameter of the follicle in mare was determined by detection of conjugated estrogens in blood (7); also, serum progesterone was evaluated before ovulation. Meanwhile, the blood steroids of pregnant (early and late gestation) in Arabian mares(8).

The growth of the dominant follicle was associated with certain intra-follicular E1-17ß and P4 levels in mares (9). Meanwhile, ovarian activity of cyclic mares was monitored by measurement of P4 and E1-17ß in plasma (5) and in follicular fluids (10) in transitional mares. The ovarian endocrine activity in the mare can be evaluated through the use of faecal steroids or their metabolities (6). Estrogens are end products of steroid metabolism and, therefore, the compounds in plasma and faeces are similar (1). Meanwhile, the faecal estrogens in relation to reproductive status in mare were demonstrated (11); they were also demonstrated in cows (12), in buffaloes (13), and in primates (14).

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Determination of the preovulatory faecal estrogens peak proved to be less successful as compared to pregnancy determination in mares (6,15). The faecal progesterone metabolites consist of several 5∝-, 5ß-pregnances and progestagens 20∝-G (1,16,17,18). They reported also that the faecal progesterone metabolites in the mare belong to the 5∝-pregnances progestagens. However, the faecal 20 ∝-hydroxyprogesterone concentration can be used to evaluate cyclic activity in mares (18). The present work aimed to monitoring the ovarian dynamics and pregnancy status using both transrectal ultrasonography and analysis of steroids in the plasma (P4 and E1-17ß) and faeces (20∝-G and E1-17ß) of Arabian mares.

Material and methods

Animals

Eight cyclic barren mares of different parities (8 – 12 years/aged), belonged to El-Zahraa Stud for Arabian horses in Cairo were used in the current work. All the mares were free from internal and external parasites and in good health condition. At the beginning of the experiments all of the mares were nonpregnant.

Methods

Heat detection was done by day after day teasing with fertile stallion. The ovarian changes during oestrus period were detected by rectal palpation and transrectal ultrasonographic examination. All mares (n=8) were naturally bred every second day from the detection of growing follicles (>20 mm in diameter) until ovulation during oestrus (19). Pregnancy was diagnosed using transrectal ultrasound scanning of the uterus at day 14 post ovulation (20), and confirmed at 21st up to 45th day later (21). The ovarian and uterine scanning were done in the pregnant mares (n=8) and nonpregnant mares (n=3) using Pie-Medical Vet-200 ultrasound with transducer 5 and 7.5 MHz (Mitsobishi inc). The pregnancy was confirmed by rectal palpation on two months post service.

Sampling

Blood and faecal samples were collected daily from all mares starting on day -7 until ovulation which is represented as day 0 (n=8). All the samples were collected daily at day 14 later (post ovulation) from all mares. Samples were collected from pregnant mares on days 21 (n=8), 28 (n=5) and 45 (n=5) after last mating. Blood samples (10 ml) were collected by jugular vein puncture into heparinized vacutainer tubes, and were centrifuged at 3000 rpm for 20 minutes. The harvested plasma was stored in portions at -20°C until hormonal analysis. The faecal samples (20 gm) were collected from rectal balls and extracted (22,23), briefly: 0.5 gm faeces mixed and vortexed in 0.5 ml water and 4 ml methanol for 30 minutes, then 3 ml petroleum ether was added and vortexed for 10 seconds. After centrifugation at 1500 rpm/15 minutes, 0.2 ml of methanol extract was transferred into a new vial then diluted with 0.6 ml distilled water and 5 ml of petroleum ether/diethyl ether (v/v 9:1). The mixture was vortexed for 30 minutes then ether was evaporated at 40°C, later on the residue was diluted with 1 ml buffer and stored at -20°C until hormonal assay.

Hormonal assay

Progesterone in plasma was assayed (8), and faecal progestagen was assayed (8,18). Progesterone was measured using sold-phase125I-progesterone RIA (Coat-A-Count Progesterone; Diagnostic Product Corporation, Los Angeles, CA, USA). The assay sensitivity was 0.07 ng/ml (rang=0.03 to 0.16 ng/ml). The intra- and inter-assay coefficients of variation were 9.0 and 9.3% respectively. While, estradiol-17ß in plasma was assayed (24,25). Estradiol-17ß was determined by RIA using (Diagnostic Product Corporation, Los Angeles, CA, USA)125I-RIA Kits. The intra- and inter-assay coefficients of variation were 9.62% and 13.43% respectively. The concentration of standard estradiol ranged between 0 to 3600 pg/ml. The assay of faecal E1-17ß was performed (1,14).

Statistical analysis

Differences between comparable groups were demonstrated with Student "t" test. All computations were done using a personal computer, with the help of statistical program SPSS/PC 3.1 of SPSS Inc.

Results

The plasma P4 and E1-17ß, also the faecal 20∝-G and E1-17ß levels during the pre and post ovulatory period are shown in Table 1. The continuous significant ($P<0.05$) increase in follicular size starting from day –7 (16.50±1.0 mm) until reaching its larger size at 0-day of ovulation (40.12±1.4 mm) was accompanied by a continuous significant ($P<0.05$) decrease in the concentration of plasma P4 and increase in the concentration of plasma E1-17ß, starting from

day –7 (0.98±0.19 ng/ml and 24.00±2.00 pg/ml, respectively). The minimum level of P4 (0.18±0.05 ng/ml), and the maximum level of E1-17ß (78.75±4.20 pg/ml) were detected at 0-day of ovulation. A similar trend was observed for the faecal 20∝-G content that show decrease in its value starting from day –7 (196.85±15.67 ng/gm) reaching its minimum level at second day post ovulation (82.67±7.29 ng/gm). Meanwhile, the faecal E1-17ß content was reaching its maximum value on day 1 after ovulation (187.50±6.27 pg/gm).

Following ovulation, the plasma levels of P4 and E1-17ß showed a continuous increase, while the faecal 20∝-G and E1-17ß showed a continuous decrease in their profiles. Meanwhile, the concentrations in plasma P4 and faecal 20∝-G increased starting from the 3rd day post ovulation, while plasma and faecal E1-17ß decreased starting from the 2nd day post ovulation.

The plasma P4 and E1-17ß and faecal 20∝-G and E1-17ß levels in pregnant and nonpregnant mares are showed in Table 2. The levels of P4 in plasma and 20∝-G in faeces were significantly (P<0.05) increased at day 14 up to day 45 of gestation than those recorded during ovulation (0-day). Meanwhile, the levels of E1-17ß in plasma and faeces was significantly (P<0.05) increased at day 21 up to day 45 of gestation than during 14th day of gestation

Table 1: Estimation the levels of progesterone and estradiol-17ß profiles in the plasma and faecal samples during pre- and post-ovulatory period in Arabian mares (Mean±S.E.)

Day related to ovulation n=8	Follicle size (mm)	Gestagens n=8		Estradiol-17ß n=8	
		Plasma P4 (ng/ml)	Faecal 20∝-G (ng/gm)	Plasma E1-17ß (pg/ml)	Faecal E1-17ß (pg/gm)
day –7	16.50±1.0	0.98±0.19	196.85±15.67	24.00±2.00	120.00±10.0
day –6	19.33±1.5	0.81±0.18	188.76±17.84	27.66±2.40	130.00±7.65
day –5	21.25±1.0	0.74±0.15	169.48±16.46	32.25±2.78	138.75±6.88
day –4	23.60±1.8	0.62±0.13	152.14±14.53	37.00±2.61	142.50±6.29
day –3	27.33±1.0	0.44±0.09	138.67±12.76	37.66±4.05	151.67±5.72
day –2	33.00±1.4	0.36±0.07	146.42±10.47	44.37±3.17	166.87±4.99
day –1	38.00±2.7	0.32±0.08	132.18±9.22	59.12±3.69	155.00±4.22
day 0*	40.12±1.4	0.18±0.05	122.34±8.73	78.75±4.20	181.87±6.81
day 1	---	0.58±0.14	118.56±8.14	40.25±2.55	187.50±6.27
day 2	---	1.16±0.35	82.67±7.29	39.25±2.82	142.50±3.78
day 3	---	1.68±0.44	136.14±13.56	35.87±1.82	122.50±2.83
day 4	---	2.17±0.58	198.25± 6.28	32.00±1.74	108.50±3.58
day 5	---	2.56±0.64	237.42±19.92	31.37±1.22	105.00±4.90
day 6	---	2.98±0.73	266.53±23.88	30.87±0.97	98.00±3.79
day 7	---	3.48±0.88	240.47±25.72	26.37±0.86	93.37±3.59
P value	P<0.05				

*day of ovulation
Means in all the columns are significantly different at level P<0.05

Table 2: Estimation the levels of progesterone and estradiol-17ß profiles in the plasma and faecal samples during early gestation period in Arabian mares (Mean±S.E.)

Day of gestation	Gestagens		Estradiol-17ß	
	Plasma P4 (ng/ml)	Faecal 20∝-G (ng/gm)	Plasma E1-17ß (pg/ml)	Faecal E1-17ß (pg/gm)
day 0* (n=8)	0.18±0.05 c	122.34±8.73 d	78.75±4.20 a	187.50±6.27 bc
day 14 (n=8)	3.86±0.82 a	440.84±31.82 b	38.37±1.94 d	163.75±16.35 c
day 21 (n=8)	4.11±0.94 a	490.36±38.48 ab	50.25±4.51 c	215.87±16.48 b
day 28 (n=5)**	5.02±0.86 a	524.56±43.12 ab	59.00±4.69 bc	285.20±19.31 a
day 45 (n=5)**	5.67±0.98 a	596.48±46.56 a	70.40±4.11 ab	317.00±22.22 a
non-pregnant day 28 n=3	1.22±0.36 b	212.63±16.68 c	33.00±1.52 e	147.33±23.67 c

*day of ovulation **3 mares were diagnosed nonpregnant
Means with different superscripts in each columns are significantly different at level P<0.05

Discussion

The hormonal profile is a reliable clinical investigation method of oestrus and pregnancy detection using analysis of progesterone and estradiol-17ß in mares (16). Meanwhile, the analysis of steroid hormones in plasma and faecal samples offer the potential of addressing many timely, integrative problems in reproduction and conservation biology (26). Our results provide evidence that plasma accompanied with faecal steroid analysis may be important for understanding the reproductive status in Arabian mares. However, the route of excretion of steroid hormones and its metabolites varies considerably among species, and also between steroids within the same species. Steroid concentrations in faeces exhibit a similar pattern to those in plasma, but have a lag time, which depending upon the species, can be from 12 to more than 48 hours (1,16). In most non-domesticated species, repeated blood sampling is not possible and, therefore, non-invasive faecal steroid evaluations are also used. Thus faecal samples are the most practicable choice beside to the plasma for this purpose.

In the present study, there was increase in follicular size starting from day -7 until reaching its larger size at day of ovulation, that accompanied by a continuous decrease and increase in the concentrations of plasma P4 and E1-17ß, starting from day –7. The minimum and maximum levels of P4 and E1-17ß reached at day of ovulation, respectively. Similarly, faecal 20∝-G content showed a decrease in its value starting from day –7 reaching its minimum level at 2nd day post ovulation, meanwhile, the faecal E1-17ß content was reaching its maximum value on day 1 after ovulation. Following ovulation, the plasma P4 and faecal 20∝-G levels showed a continuous increase (starting from the 3rd day post ovulation), and decrease in the profiles of plasma and faecal E1-17ß (starting from the 2nd day post ovulation). With increasing the follicular size from <30 mm to >30 mm diameter, there was a significant increase in the concentration of plasma E1-17ß and decrease in the concentration of plasma P4 (27). However, production of estrogen by the large follicles is consistent with the oestrus-like uterine echotexture which seemed approximately related to the growing phase of large follicle (7). A high relation between the ultrasonography findings and hormonal concentration, showing the increase of E1-17ß and the decrease of P4 concentration, corresponding to the days of the oestrus cycle at which the experiments were performed (2,28). In addition, the incidence of diestrous ovulations in mares is considerably higher (29), presumably because some breeds have more follicular activity and secretion of estrogen during the first half of dioestrus. This come in agreement with the findings in this study where the secretion of E1-17ß extended up to the second day post ovulation. Results from faecal hormone analysis indicated a useful characterizing and retrospectively predicting oestrus cyclicity and the occurrence of ovulation. Furthermore, cyclicity and ovulation were also confirmed by the rise and fall of the progestagens and E1-17ß excretion during the pre- and post-ovulatory periods (26).

For the study of ovarian activity in mares, several investigators have measured the concentration of

P4 in blood (30,31,32). There is agreement that concentrations below 1 ng/ml plasma (33) are indicative for oestrus or missing luteal activity. After ovulation, the values of P4 increase within 24-36 h, and remain high until day 14 or 15. Thereafter, in nonpregnant mares the values decrease rapidly to the low oestrus values. The plasma P4 and E1-17ß concentrations were similar to those found by others in the late luteal and follicular phases of the oestrus cycle of the mare (31,32,34). Moreover, large quantities of steroids are excreted in faeces largely because the principal means of excreting cholesterol (the progenitor of most steroids) is through the gastro-intestinal tract via bile (35). For this reason, some steroids and their metabolites may be excreted in faeces at concentrations that reflect biological events. The previous results indicated that the excretion of steroids into the gut is mainly through bile (1), but they have also shown that a small proportion of the circulating steroids is secreted through the mucosa of the large intestine (36). Furthermore, steroids might be unevenly distributed in the faecal balls of horses (37).

The levels of P4 in plasma and 20∝-G in faeces was significantly increased at days 14 up to 45 of gestation than those recorded during at ovulation in nonpregnant mares. Meanwhile, the levels of E1-17ß in both plasma and faeces was increased at days 21 up to 45 of gestation than those estimated during 14th day of gestation. However, faecal progesterone metabolites and estrogen determination proved to be reliable indicators for pregnancy diagnosis in the species in which the foeto-placental unit is the source of large quantities of estrogens (1). The differences in these two variables between pregnant and nonpregnant mares reflect the first luteal response to pregnancy and could be an expression of the maternal pregnancy recognition mechanism (3). During the oestrus cycle and pregnancy, P4 is produced by corpus luteum and its metabolities circulated in the peripheral plasma and may be excreted via faeces (38), that could be used for monitoring the growth, maintenance and regression of corpus luteum, and thus, as a tool to confirm oestrus cyclicity and possible pregnancy. However, faecal progestagen values increased at luteal phase within 10 days after fertilization and remained in this range for the first 2 months of pregnancy (17). Likewise, plasma P4 concentrations were measured in 179 mares bled on alternate days commencing with a positive pregnancy diagnosis on day 17 to 18 after ovulation and concluding on days 42 to 45 (40). Similar to our findings, faecal progestagen analysis has been successfully used for monitoring corpus luteum function and pregnancy (14,16,23,41). Although some studies reported the determination of the preovulatory oestrogen peak in mares, these methods proved to be less successful as compared to pregnancy determination, peak concentrations of faecal estrone conjugates during the follicular phase was very low (6,15). So, for a reliable analysis of the preovulatory estrogen peak in faecal samples, more extraction and clean up procedures of the samples and sensitive assays would be necessary. Moreover, follicular waves occurred periodically until the corpus luteum regressed, and in the absence of luteolysis (pregnant mares) the periodicity continued (42).

The difference in the excretion time of steroids between the oestrus cycle and pregnancy is probably caused by the very high concentrations present during pregnancy and by the enterohepatic circulation, which retards the excretion (16,17). Subsequently, the differences between the concentrations of both P4 and E1-17ß during oestrus and early gestation period could predicate the reproductive status of the mare. Subsequently, more research and coordination between researchers and biotechnology industries are required before any on-farm or field type faecal progestagen kits can be developed.

In conclusion, plasma and faecal steroid analysis can be used and accepted as a reliable and a diagnostic tool to study the fundemental reproductive endocrinology and provide information regarding the oestrus cycle and early pregnancy. Meanwhile, the E1-17ß and progesterone metabolites might be more accurate for monitoring the reproductive performance of mares. Subsequently, the ultrasonography accompanied with the estimation of steroid levels in plasma and faeces has a predictive value for the assessment of follicular sizes, ovulation time and early pregnancy in Arabian mares.

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PROFIL STEROIDNIH HORMONOV MED ESTRUSOM IN ZGODNJO BREJOSTJO PRI ARABSKIH KOBILAH

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Povzetek: Za ugotavljanje reproduktivnega ciklusa pri kobilah predstavlja ovrednotenje steriodnih hormonov v krvni plazmi in blatu že ustaljeno metodo. Namen naše študije je bil primerjati vrednosti gestagenov in estradiola 17ß v plazmi in blatu s spremljanjem sprememb na jajčnikih in maternični sluznici s transrektalno ultrazvočno preiskavo pri arabskih kobilah. Študija je zajela 8 kobil arabske pasme z normalnim ciklusom. Hormone smo določali radioimunsko. Ugotovljeno rast folikov je spremljal statistično značilen (P<0.05) padec gestagena P4 in porast estradiola 17ß (E1-17ß) v krvni plazmi. Poleg tega smo ugotovili najnižjo raven P4 in najvišjo E1-17ß na dan 0 – čas ovulacije. Podobno je progesteronski metabolit v blatu 20∝-hydroxy-progesterone (20∝-G) kazal statistično značilen (P<0.05) padec ravni od sedmega dneva pred ovulacijo z najnižjo vrednostjo dva dni po ovulaciji, medtem ko je E1-17ß v blatu dosegel najvišjo vrednost 1 dan po ovulaciji. Po ovulaciji se je od tretjega dne dalje vrednost plazemskega P4 in fekalnega 20∝-G konstantno statistično značilno (P<0.05) poviševala, medtem ko se je vrednost estrogena v plazmi ali blatu konstatno zniževala. Med brejostjo sta v blatu statistično značilno narasli vrednosti P4 20∝-G od 14. do 45. dneva v primerjavi z dnevom 0 pri nebrejih kobilah. Raven E1-17ß je značilno narasla (P<0.05) v blatu in krvni plazmi med 21. in 25. dnem brejosti, če jo primerjamo z vrednostmi v prvih dveh tednih brejosti pri brejih oz. pri nebrejih kobilah. Ugotovimo lahko, da so vse uporabljene metode, kot so ultrazvočna preiskava, merjenje P4 in E1-17ß v plazmi ter 20∝-G in E1-17ß v blatu, uporabne za napovedovanje velikosti jajčnega folikla, čas ovulacije in ugotavljanje zgodnje brejosti pri arabskih kobilah.

Ključne besede: arabske kobile; estradiol-17ß; gestageni; ultrazvočna preiskava; estrus; brejost

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Original Research Paper

SOME MORPHOLOGICAL ASPECTS OF THE CREMASTER MUSCLE IN BROWN HARE AND DOMESTIC RABBIT

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Summary: The cremaster muscle is formed by muscle fibres that are descended from the caudal border of *m. obliquus internus abdominis* in males. The extent and functioning of the cremaster muscle differs among mammals. In rodents and lagomorphs testes migrate between scrotum and abdominal cavity and therefore the cremaster is extensive and highly active. There were hypotheses that the functioning of cremaster muscle is correlated with the seasonal testicular regression in brown hare. We previously established that position of the testes in hares is strongly correlated to the ambient temperature and not to the reproductive activity. Our present aim was to establish fibre type profile of the cremaster muscle in comparison with its origin, the internal oblique abdominal muscle, in both brown hare and laboratory rabbits. Muscle samples from 6 hares (3 in quiescent and 3 in reproductively active period) and 6 rabbits were collected and subjected to enzyme histochemistry (fibre typing according to mATPase, SDH and α-GPDH activity and glycogen content) and immunohistochemistry directed to different myosin heavy chain isoforms. The cremaster muscle completely surrounded the testicle and epididymis in both species. While enzyme- and immunohistology revealed *m. obliquus internus abdominis* as a predominantly fast muscle, central bundles of cremaster muscle contained mainly slow fibre types. However, the peripheral parts of cremaster were predominantly composed of fast IIA fibre type indicating functionally important intra-muscular variability. In rabbits, which were housed in constant ambient temperature, the central "slow" bundle was substantially smaller. Furthermore *m. cremaster* had high glycogen content and high glycolitic capacity, including the presence of uncommon SOG fibres. No prominent differences in fibre types between breeding and non-breeding seasons were found.

Key words: *m. cremaster*; brown hare; rabbit; skeletal muscle fibres; mATPase; myosin

Introduction

In rodents and lagomorphs testes can freely migrate between scrotum and abdominal cavity because of open inguinal canal. Abdominal displacement is important in regulating intratesticular temperature (in favourable nutritional conditions most rodents breed also in the coldest months), protecting testes in fights and also slightly helping the ejaculation (1). *M. cremaster* is extensive and likely active muscle. Some rodents (i.e. chinchillas, degus) are referred to as nonscrotal rodents, where testes remain inside abdomen and only tail of epididymis lays inside scrotal sac (2). Lagomorpha seem to lie further apart, being phylogenetically more close to primates than rodents, however, they still share many physiological and anatomical features with rodents (3). Scrotal sac is doubled and clearly evident in descended testis. In laboratory rodents, the cremaster muscle consists of fairly thin fleshy bundle arranged in a manner to form a sac that encloses the testicle. The muscle is usually referred as an extension of *m. obliquus abdominis internus* but in rat and human also contribution of *m. transversus abdominis* is reported (4, 5, 6). Despite *m. cremaster* being used in physiological experiments and experimental surgery for decades (7), exact morphological aspects or the fibre type profile of the cremaster muscle is not widely known among mammals. In rat and hamster the reported predominant fibre type (60-80% of the muscle area) was IIB according to Brooke and Keiser classification at the time – today's methods would probably demonstrate the majority of IIX fibres. Besides, the number of those

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fibres decreased with age in favour to IIA fibres (8). In contrast, in carnivore ferret the type I fibres predominated with 66.2% (9). In guinea pigs both slow and fast fibres were reported but no details on ratio was given (10). In a comparative study of domestic and laboratory mammals the highest proportion of proprioreceptors (muscle spindles) in cremaster were found in rabbits, followed by sheep and rodents (11) indicating an active muscle in species we decided to research.

In previous research on brown hare reproductive activity we found a strong correlation between the testis location and ambiental temperature. In coldest months, the testes were displaced abdominally halfway or totally, regardless of the current reproductive activity – full or arrested spermatogenesis (12). Therefore we assumed that *m. cremaster* must be specifically adapted to special functioning and the fibre type profile would differ from it's origin – *m. obliquus abdominis* internus. Differences in fibre types between breeding and non-breeding season (or warm and cold months) were expected in brown hare, but not in domestic rabbit, bred in constant laboratory conditions. The aim of this study was therefore to evaluate the position and extent of *m. cremaster* and to establish basic fibre type profile in brown hare and domestic rabbit.

Material and methods

Muscle samples

The muscles were sampled from 6 brown hares (*Lepus europaeus*) bred in outdoor pens. 3 were sacrificed in reproductively active period (July) and 3 in quiescent period (November). About 1 cm ring of *m. cremaster* was cut on three locations: proximal part (at the point of attachment to tunica vaginalis), middle part (at the head of epididymis) and lower part (at the tail of epididymis). 1 cm^2 of m. obliquus internus abdominis was also taken from most caudal border of the muscle, where the distinction between abdominal wall and scrotal sac becomes evident.

For comparison purposes cremaster and internal oblique abdominal muscles from 6 conventional laboratory rabbits (New Zealand white rabbit) were also taken. They share very similar anatomy with brown hare, including the location of reproductive organs and muscles. The animals were housed at constant ambient temperature of 18°C.

Muscle samples were frozen with submersion in liquid nitrogen and stored at -80°C. Transverse serial cryosections (10 μm) were cut on Leica CM 1800 cryostat at -17°C, mounted on 3-aminopropyl triethoxysilane (APES) covered slides and air-dried.

mATPase histochemistry

To determine fibre types the sections were processed for mATPase reaction following the procedure by Brooke and Keiser (13). The sections were incubated in 0.1 M Na-acetate at pH 4.35 and in 0.2 M Na-acetate at pH 4.4 and 4.6 for 5 minutes at room temperature. For the alkaline preincubation, the solutions of 0.1 M $CaCl_2$, 0.07 M Na-acetate and 0.075 M Na-barbital adjusted to pH 9.8 and 10.2 were used (15 minutes, room temperature). After preincubation sections were washed and incubated in solution of 0.1 M CaCl2, 0.07 M Na-acetate and 0.075 M Na-barbital, pH 9.45 with addition of 1.5 mg/ml of ATP for 60 minutes (following the acid preincubation) or 30 minutes (following the alkaline preincubation) at 37°C. For fibre type designation the described results by Hämäläinen and Pette (14) were observed.

Immunohistochemistry

To demonstrate the myosin heavy chain (MHC) isoform expression, different primary monoclonal antibodies specific to MHC isoforms of different mammals were used. MHC-I was revealed with antibody MHC-slow (diluted 1:40 in PBS) supplied by Novocastra Laboratories. To demonstrate the expression of fast MHC isoforms MHC-fast (diluted 1:40 in PBS; Novocastra Laboratories), A4.74 (diluted 1:40 in PBS, Alexis Biochemicals) specific to MHC-IIa in man and rodents but also reacting with MHC-IIx in dog (15), BF-F3 directed against MHC-IIb and BF-35, an antibody specific to all MHC isoforms except MHC-IIx (16) were used. Serial cryosections were air-dried, washed with phosphate buffer saline (PBS) and then incubated with primary antibody in humidified box overnight at 4°C. Novostain Super ABC Kit (Novocastra Laboratories) was used to reveal immunohistochemical reaction according to the manufacturer's instructions. The stained sections were then dehydrated and mounted with Syntetic Moutant (Shandon, USA).

Metabolic profile of muscle fibres

To estimate the fibres' basic metabolic profile the activity of the oxidative enzyme succinate dehydrogenase (SDH) and the glycolytic one, i.e. mitochondrial menadion-linked α-glycerophosphate dehydrogenase (α-GPDH) was demonstrated as previously described by Nachlas et al. (17) and Dubowitz and

Brooke (18), respectively. Glycogen content in the fibres was demonstrated with PAS staining (Periodic acid-Schiff reaction).

Serial cryosections were analyzed with Nikon Microphot FXA microscope (Nikon instruments Europe B.V., Badhoevedorp, The Netherlands) and Lucia-G image analysing system (Laboratory Imaging, Prague, Czech Republic).

Results

The position and extent of m. cremaster

In scrotally located testes, that is in all rabbits and hares from summer, the cremaster muscle was thin, practically translucent layer, completely encircling processus vaginalis (Fig 1a). On dorsal aspect, especially in the pampiniform plexus region, the muscle was darker to naked eye - seemingly thicker (Fig 1b). This part originated on and parallel to *lig. inguinale* (Fig 1c). In retracted testis (hares from November) the cremaster was a reddish creased sac, about 1 centimetre long still encircling the *cauda epididymis* (Fig. 2).

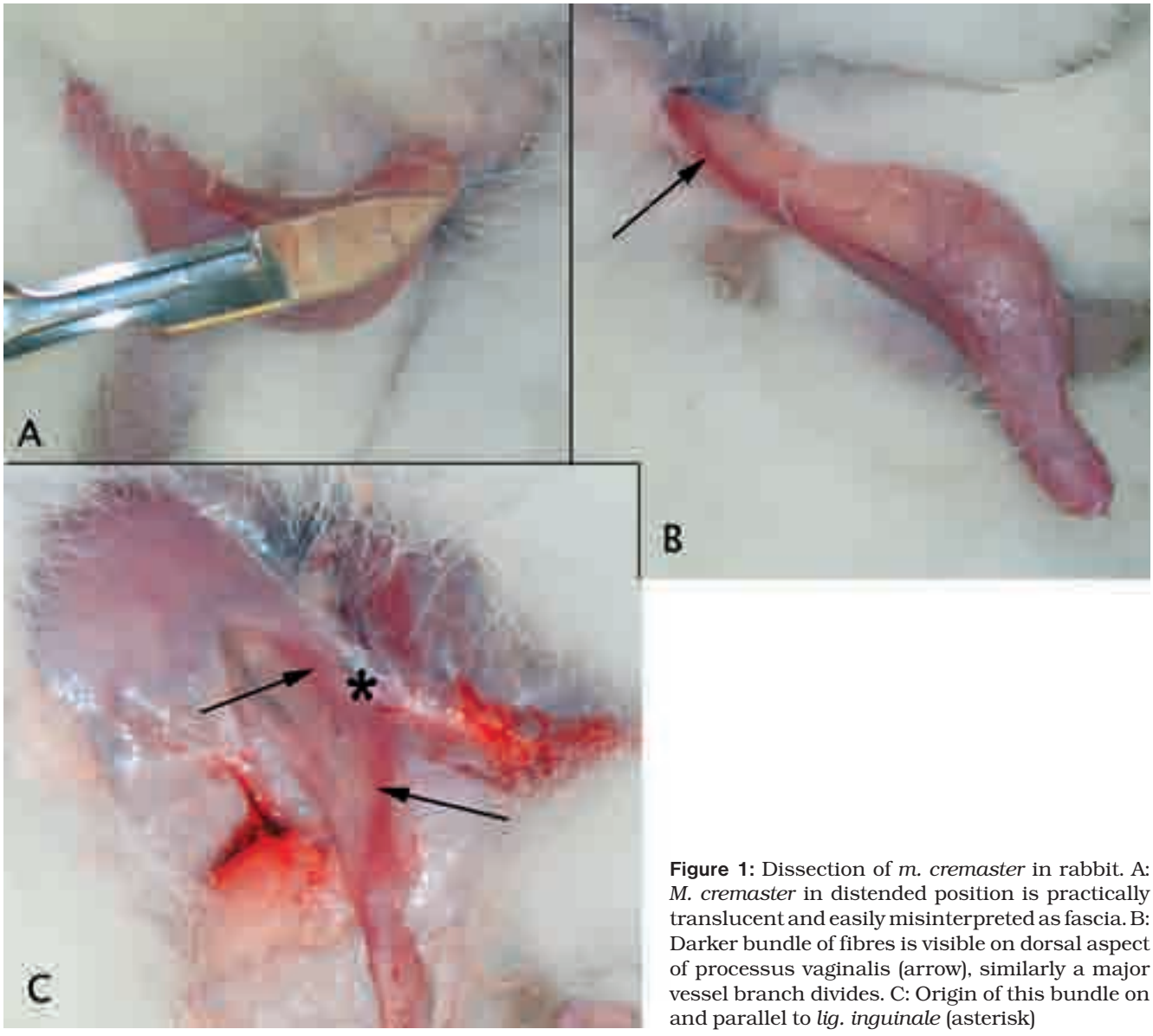

Figure 1: Dissection of *m. cremaster* in rabbit. A: *M. cremaster* in distended position is practically translucent and easily misinterpreted as fascia. B: Darker bundle of fibres is visible on dorsal aspect of processus vaginalis (arrow), similarly a major vessel branch divides. C: Origin of this bundle on and parallel to *lig. inguinale* (asterisk)

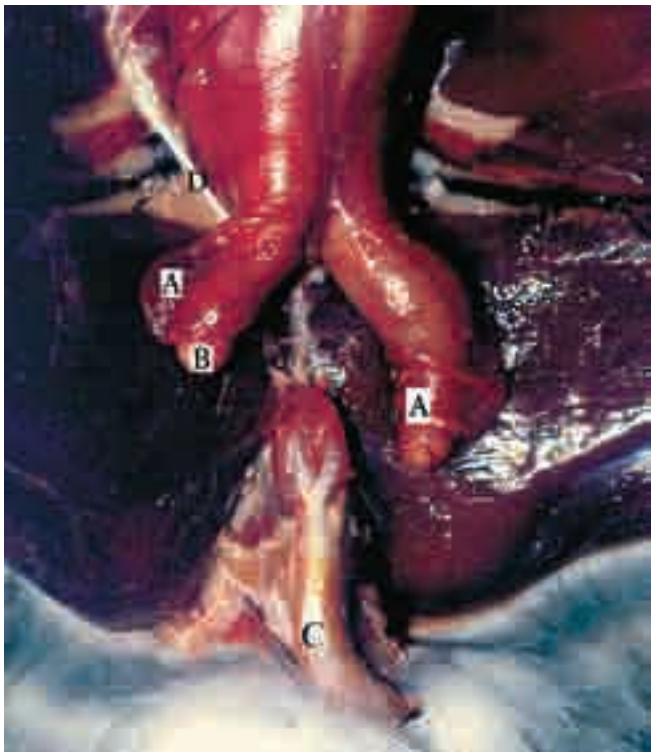

Figure 2: Inguinal and abdominal displacement of testes during cold months in brown hare. Skin removed. *M. cremaster* (A) is visibly thick and creased over the *cauda epididimis* (B). Penis (C), *lig. inguinale* (D)

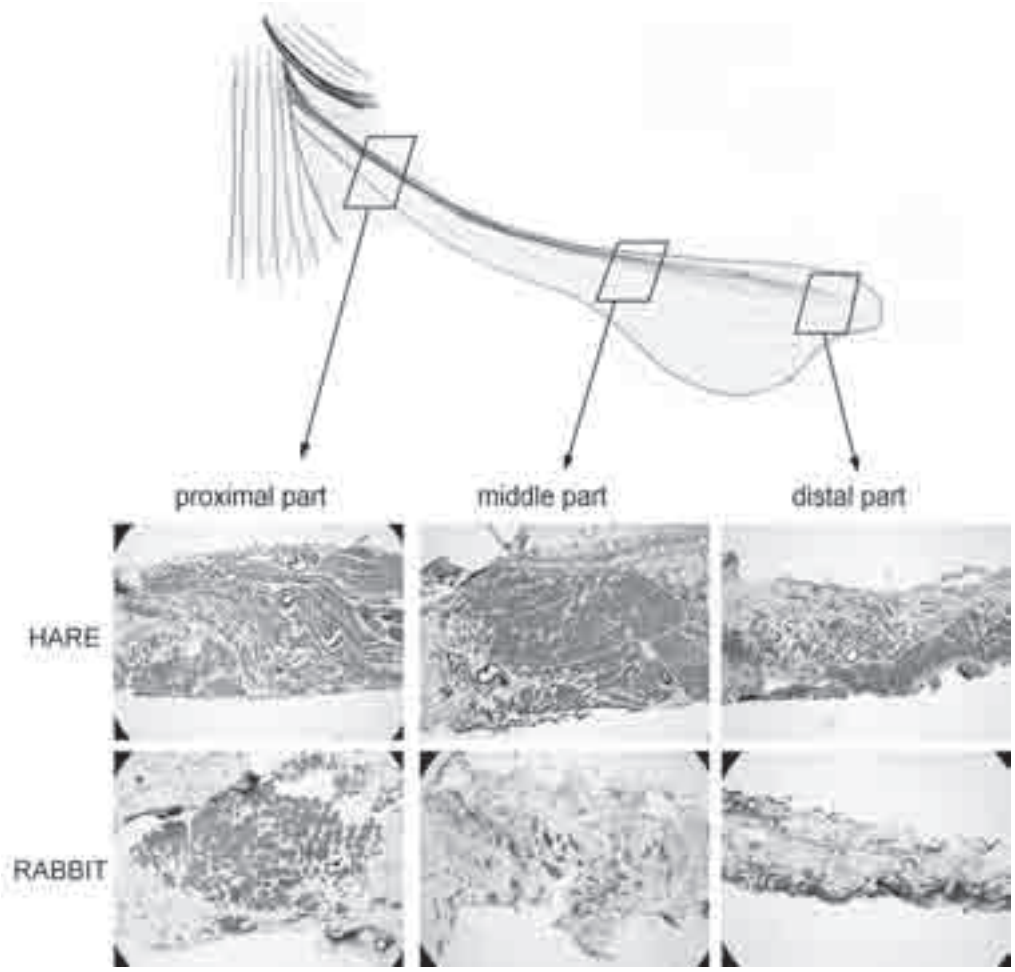

Figure 3: Cross sections of cremaster muscle on three different locations, as graphically represented above, at small magnification shoving the region of dorsal muscle aspect. Muscle fibres in this region stained predominantly dark (slow fibres) in mATPase reaction with acid preincubation in hare. In rabbit such bundle was evident only in proximal region of muscle and became superficially located in distal parts

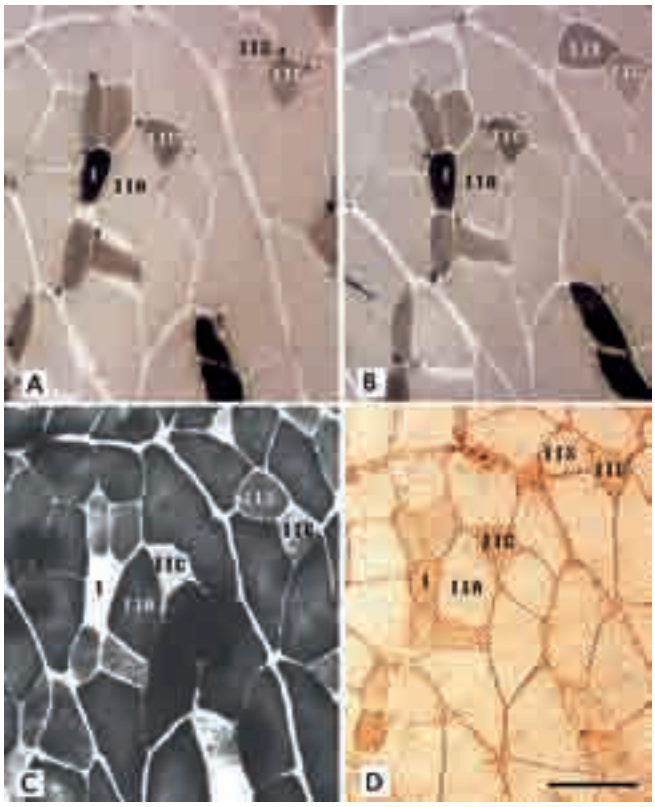

Figure 4: Serial cross-sections of *m. obliquus internus* abdominis in rabbit. Fibre types I, IIC, IIA and IIX were distinguished. A: staining for mATPase after preincubation at pH 4.3, B: 4.5, C: 10.2, D: immunohistochemistry with MHC-slow antibodies. Scale bar = 100 μm

Fibre types in m. cremaster and m. obliquus abdominis internus

Based on mATPase typing and immunohistochemistry demonstration of slow and fast isoforms fibres I, IIC, IIA, IIX and IIAX were demonstrated. Antibody A4.74 gave the same results as MHC-fast antibody (positive in IIA, IIAX and IIX and weakly positive in IIC) and this agreed with mATPase typing and MHC-slow reactions. No clear positive reactions in staining with BF-F3 were found therefore no IIB fibres were demonstrated. Also no clear negative reaction with BF-35 antibodies was encountered (results not shown) therefore this antibody is not very specific to distinguish between IIA, IIAX and IIX fibres in rabbit or hare.

The *m. cremaster* was not homogenous. It was clear that the central portion, as graphically represented in Figure 3, predominantly contained different fibre types than peripheral parts. This central congregation of predominantly slow fibres corresponded to dorsal aspect of vaginal process and was extensive in hare, but in rabbit pronounced only in the proximal part, while distally the slow fibres remained congregated superficially in the dorsal aspect (lower right in Fig. 3). Peripheral parts had high counts of fast IIA fibres, comparable to *m. obliquus abdominis* internus (Fig. 4) and even higher – there were no slow fibres at all in some distal and peripheral fascicles. Bundle of slow fibres normally didn't have distinct boundaries – the transitional area had mixed count of fibres (Fig. 5). Fibre diameter differed between the two muscles – in *m. obliquus abdominis* internus the average diameter was close to 50 μm, in *m. cremaster* 25 μm.

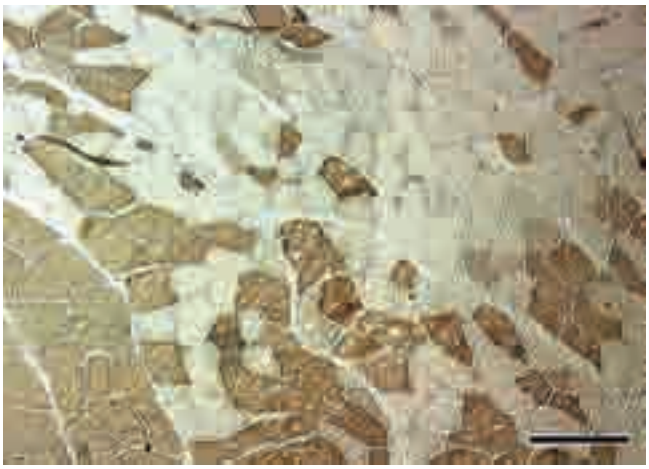

Figure 5: Section of transitional area between bundles of exclusively type I fibres (dark staining – lower left) and other parts of the muscle that contain predominately type IIA fibres (weak staining - upper right) in *m. cremaster* of brown hare. mATPase histochemistry, pH 4.5. Scale bar = 100 μm

Further distinction between rabbit and hare was evident in the number of hybrid fibres, which were scarce in cremaster of rabbit (data not shown) but averaged 15% in cremaster of hare, especially type IIC (Figure 6); a few IIAX fibres were also noted. Interestingly, in both animals all muscle fibres had a pronounced glycolitic capacity (α-GPDH); the enzyme-histochemistry staining intensity varied moderately in rabbit but only slightly in hare. A classification of SOG (slow oxidative-glycolitic), FOG (fast oxidative-glycolitic) fibre types could be applied (Figure 7). Compared to *m. obliquus internus abdominis* the cremaster muscle also had substantively higher glycogen content in the muscle fibres as established with PAS staining (Fig. 8). No significant variations in fibre type proportions between breeding and non-breeding season were found in hare.

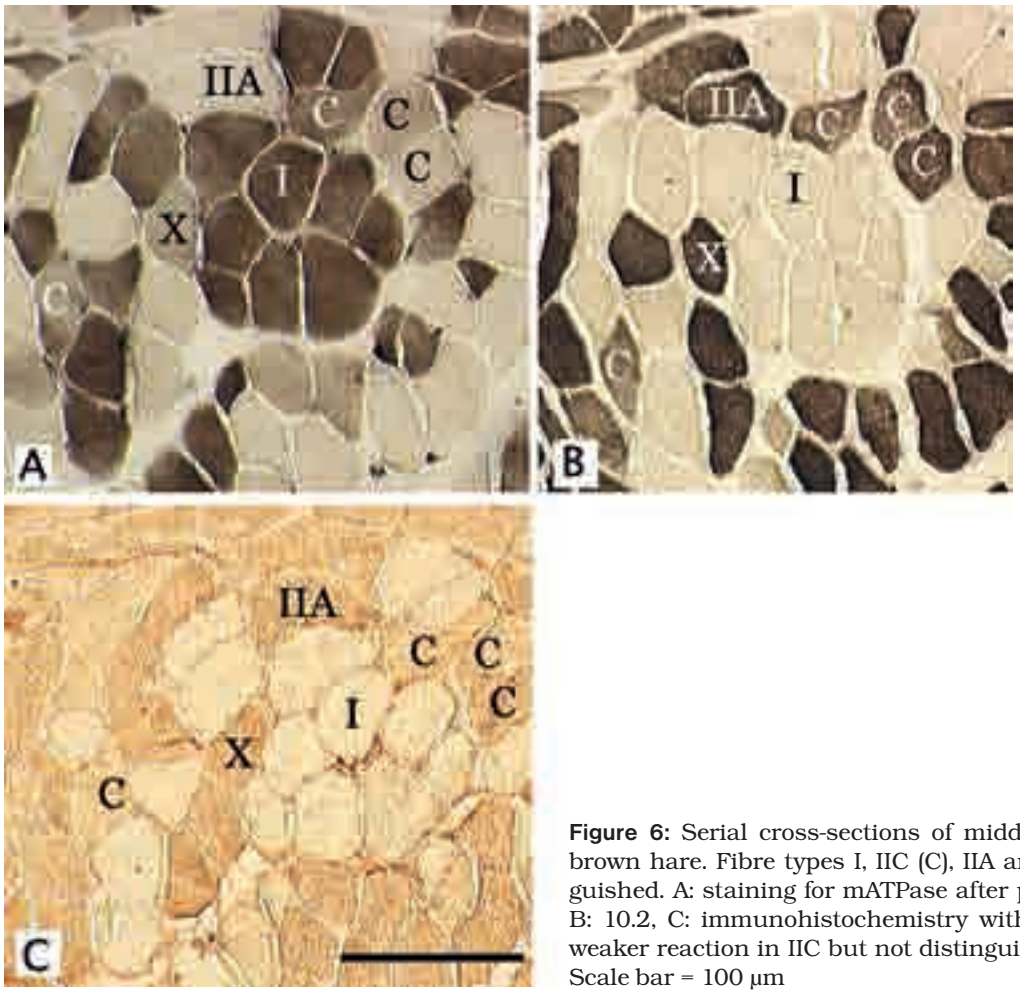

Figure 6: Serial cross-sections of middle part of *m. cremaster* in brown hare. Fibre types I, IIC (C), IIA and IIX (X) fibres are distinguished. A: staining for mATPase after preincubation at pH A: 4.5, B: 10.2, C: immunohistochemistry with A4.74 antibody (showing weaker reaction in IIC but not distinguishing between IIA and IIX). Scale bar = 100 μm

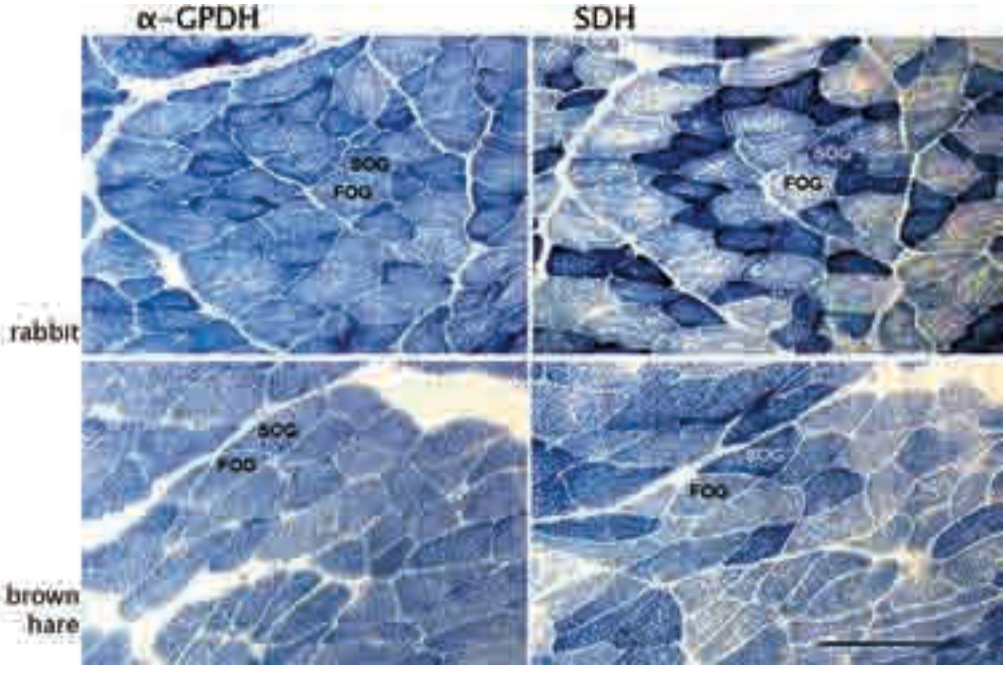

Figure 7: Enzyme histochemistry for α-GPDH and SDH in *m. cremaster* of rabbit and hare in mixed fibres area. Glycolitic capacity is high in all fibres, although variation is more evident in rabbit. Comparing with mATPase or immunohistochemistry one can determine mainly SOG and FOG fibres. Scale bar = 100 μm

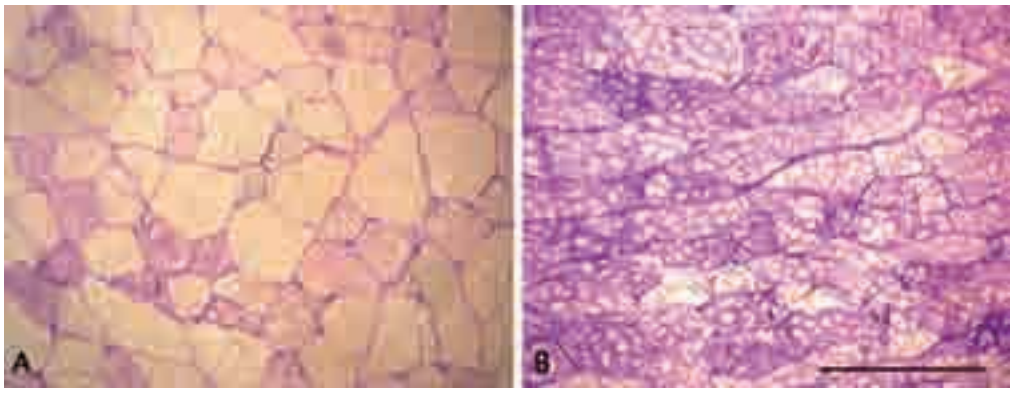

Figure 8: PAS reaction in *m. obliquus internus abdominis* (A) and *m. cremaster* (B) in the same brown hare. Scale bar = 100 μm

Discussion

The results of our study clearly indicate that *m. cremaster* is adapted to special functioning, since it differs from its origin, the caudal border of *m. obliquus abdominis* internus. It has different fibre type profile – the central bundle of *m. cremaster* consists almost exclusively of type I fibres, all fibres have pronounced glycolitic capacity and fibres throughout the muscle have a smaller diameter than those in abdominal muscle. We also noted the cremaster muscle is quite well developed. In widely used anatomical atlases by Popesko (19), the sketch of *m. cremaster* in rabbit is rather misleading. It depicts cremaster as a thin muscle bundle, limited to the dorsal aspect of tunica vaginalis and extending until head region of the testis. As expected, in all dissected animals the cremaster muscle composed a complete pouch. The name of cremaster in Slovene language literarily means the "lifter of the testis" but in lagomorphs (and rodents) when contracting, the effect would not be pulling the tunica vaginalis with testis towards abdominal cavity but rather squeezing out the scrotal sac, like contents of a tube.

Occasionally the textbooks and manuals refer to the cremaster muscle as *m. cremaster externus* as opposed to *m. cremaster* – smooth muscle fibres associated with connective and vascular components of spermatic cord, which are not present in all mammal species neither in all individuals. Due to the structure of the latter (smooth muscle) such terminology is also misleading and should be abandoned.

Although the rabbit muscles regularly express 3 fast MHC: IIa, IIx and IIb (20), in cremaster muscle the used antibodies did not demonstrate MHC-IIb or MHC-IIx fibres, at least not in a pure form. The IIB fibres were also not demonstrated with mATPase reaction considering the classification described for rabbit (14), but the antibody unspecifity must be taken into consideration at least for demonstrating IIx isoforms. Antibody A4.74, although according to manufacturer directed against MHC-IIa in rat, mouse, humans and rabbit, obviously cross reacts with IIx isoform in many species, as was noted before (15, 21). Small amounts of faster MHC may be present , but even if detected with other methods, it is clear that their functional importance is not significant.

Considering the fibre type composition we found that hares had a higher percentage of slow fibres along the length of cremaster muscle than rabbits. This speaks of greater adaptation of muscle to specific function in hare. But both species exhibited intramuscular variations – different central and peripheral parts of the muscle. The peripheral parts, consisting of predominantly IIA fibres, were more extensive in rabbit. Because these two parts were not clearly demarked (a transitional area with mixed fibres existed) we did not perform quantitative analysis of fibre types. While one can determine much higher content of oxidative enzyme in slow muscle fibres compared to fast, there is unusually high reaction to glycolitic enzyme content, almost not distinguishable between fibre types in brown hare. We determined SOG fibres (type I fibres with high SDH and α-GPDH) which are rarely mentioned in the literature as it is expected that slow fibres do not have active glycolitic enzymes. The prominent activity of α-GPDH in our case might be a peculiarity of *m. cremaster*.

We have two hypotheses how the central part of *m. cremaster* on dorsal aspect of vaginal process, which was thicker and had a distinctive fibre composition, is formed. It can represent fibres, which are descended from different muscle as the rest of *m. cremaster* i.e. *m. transversus abdominis* like in rats or man (4, 5, 6). But no clear appositions (transverse against circular) were found in rabbit or hare in selected transverse sections nor thicker perymisium separating the bundles. A detailed micro-dissection

should be performed to confirm or reject uniform distribution of muscle fibres in these species.

On the other hand, different innervations can be the cause of different immuno- and enzyme-histochemical characteristics. In rat a small nucleus in ventral columns of lumbar segments L1 and L2 was demonstrated to innervate cremaster muscle (22). In fact, the femoral branch of genitofemoral nerve innervates first caudal portions of transversus and oblique internus abdominis and initial part of cremaster, and then disperses into dorsal, ventral and most notably lateral face of cremaster muscle. Genital branch of genitofemoral nerve innervates abdominal-inguinal skin as well dorsal, ventral and medial parts of cremaster. For innervation of transitional muscle region that may represent kind of cremasteric sphincter, also ventrolateral nucleus is responsible (5). Therefore we can suppose that nerve fibres contribution from the two different motoneuron regions might be the case in lagomorphs.

In either case, the consequence is the physiology. The central part of *m. cremaster*, located on the dorsal aspect of processus vaginalis, seems responsible for a sustainable pull of testes during the cold months since it contains predominantly slow fibres. The imminent cremasteric reflex (in stress, fights) would be achieved by the rest of the muscle (fast, but fatigue resistant IIA fibres). This supposition can be supported by the fact that in rabbit the central slow fibre part of the cremaster was narrower and shorter, as those animals were not subjected to cold ambient temperatures. More numerous hybrid fibre types in hare also indicate muscle plasticity – animals living in wild had higher capacity to adapt to environmental conditions as laboratory animals.

Surprisingly however, no significant differences between muscle fibre type profile was found in hares between warm and cold months and it seems *m. cremaster* is highly responsive throughout the year regardless of reproductive state.

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NEKAJ MORFOLOŠKIH ZNAČILNOSTI MIŠICE VZDIGOVALKE MOD (*M. CREMASTER*) PRI POLJSKEM ZAJCU IN DOMAČEM KUNCU

M. Štrbenc, G. Fazarinc, A. Pogačnik

Povzetek: Mišica vzdigovalka mod (*m. cremaster*) pri samcih izvira iz mišičnih snopov kavdalne meje notranje poševne trebušne mišice (*m. obliquus internus abdominis*). Med sesalci so opazne razlike v velikosti in delovanju vzdigovalke mod. Pri glodavcih in lagomorfih se moda prosto premikajo med mošnjo in trebušno votlino, zato je vzdigovalka mod obsežna in dejavna mišica. V preteklosti so domnevali, da je njeno delovanje pri teh vrstah živali povezano s sezonskim nazadovanjem mod, vendar smo v prejšnji raziskavi ugotovili, da je položaj mod odvisen od temperature okolja in ne od paritvene sezone. Namen te študije pa je bil ugotoviti profil mišičnih vlaken v vzdigovalki mod v primerjavi z izvorno, notranjo poševno trebušno mišico pri poljskem zajcu in domačem kuncu. Mišične vzorce smo vzeli šestim zajcem (3 iz paritvene sezone in 3 izven nje) in šestim kuncem. Histološke rezine mišic smo obarvali po postopku encimske histokemije (klasifikacija mišičnih vlaken po metodi mATPaze, aktivnosti encimov SDH in α-GPDH ter vsebnosti glikogena) in imunohistokemično za različne izoforme težkih verig miozina. Ugotovili smo, da vzdigovalka mod popolnoma obkroža modo in nadmodek pri obeh vrstah živali. Na histoloških rezinah smo ugotovili, da zadnji del notranje poševne trebušne mišice po profilu vlaken predstavlja hitro mišico. Nasprotno pa je vsaj osrednji del vzdigovalke mod vseboval predvsem počasna vlakna, ki v perifernih delih mišice nadomestijo vlakna IIA. Vzdigovalka mod torej ni homogena mišica. Pri kuncih, ki so bili nameščeni v okolju z nespremenljivo temperaturo, je bil osrednji »počasni« snop vlaken bistveno manjši oz. krajši kot pri zajcu. V vzdigovalki mod smo ugotovili še visok nivo glikogena in visoko glikolitično kapaciteto vključno s t. i. vlakni SOG (počasi krčljiva oksidativno-glikolitična). Med vzorci mišic iz paritvene sezone in izven nje ni bilo razlik.

Ključne besede: *m. cremaster*; poljski zajec; kunec; skeletna mišična vlakna; mATPaza; miozin

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Case Report

DEVELOPMENT OF AN ANIMAL HEALTH SURVEILLANCE INFRASTRUCTURE IN BOSNIA AND HERZEGOVINA – CASE REPORT

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Summary: Since 1996, Bosnia and Herzegovina has made significant efforts to enhance its national veterinary infrastructure in order to improve animal health, public health, and food safety. Many international agencies and donors have invested substantial funds to rebuild the livestock industry which was devastated during the last war (April 1992 - November 1995). There have also been significant efforts to improve veterinary services in the country. However, improvements only became apparent after the State Veterinary Administration for Bosnia and Herzegovina was established in December 2000. Recent World Trade Organization accession negotiations and efforts to comply with Sanitary-Phytosanitary agreement have underscored the need to further address animal health and disease issues at a national level in Bosnia and Herzegovina.
The Animal Health Economics Center of the Veterinary Faculty in Sarajevo has collaborated with the Animal Population Health Institute of Colorado State University since 2001. Since this cooperation began, many activities have been initiated to develop and establish an effective national veterinary infrastructure. Training programs and technical workshops supported through United States Department of Agriculture funding have been organized in Bosnia and Herzegovina, and topics covered to date include: animal health control programs, surveillance, national disease prioritization, food safety, and the initiation of regional cooperation. National surveillance systems are now being developed and implemented for diseases targeted as a result of a disease prioritization workshop.

Key words: Bosnia and Herzegovina; veterinary service development

Introduction

To fully understand agriculture production in Bosnia and Herzegovina (BiH) we must bear in mind the enduring consequences of the war (April 1992 - November 1995). These include a substantial decrease in the human population, massive outward migration and widespread social problems related to refugees. BiH is also a country in transition from a communist regime and suffers substantial weaknesses in public administration, taxation and its general economy. The Dayton peace agreement did not provide a legal framework for a ministry of agriculture at the state level and instead delegated the responsibilities for most of governmental functions including agriculture to its two Entities, the Federation of Bosnia and Herzegovina (FBiH) and Republic of Srpska (RS) and the independent district of Brcko (BD). This, coupled with a distinct lack of coordination between its three parties, has presented a major handicap to the country's development during the post war period (1).

With respect to veterinary services, the lack of a central level administration and a national disease control and surveillance plan during the period from 1995 to 2003 created negative consequences on the animal health situation and isolated the country

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from regional and international markets as well. Reliable animal disease data were almost non-existent during the immediate post-war period (2). Initially, disease information was passively acquired, and collection was sporadic and most commonly initiated in response to public pressure. The usual response to a disease outbreak was through a policy of test and removal of sero-positive animals, but even this was hampered by a lack of sufficient funding.

Improvement in animal disease surveillance and control only became possible once the State Veterinary Administration (SVA) for BiH was established in December 2000 (3). However, the SVA became effective only after a state veterinary law was adopted in October, 2002 (4). Recent World Trade Organization (WTO) accession negotiations and efforts to comply with Sanitary-Phytosanitary (SPS) agreement have underscored the need to further address animal health issues at a national level in BiH.

The Animal Health Economics Center of the Veterinary Faculty in Sarajevo has collaborated with the Animal Population Health Institute (APHI) of Colorado State University since 2001. Since this cooperation began, several activities have been initiated to develop and establish an effective national veterinary infrastructure. Training programs and technical workshops supported through United States Department of Agriculture – Animal and Plant Health Inspection Service (USDA-APHIS) funding have been organized in BiH, and topics covered to date include: animal health control programs, surveillance, national disease prioritization, food safety, and the improvement of regional cooperation. National surveillance systems are now being developed and implemented for targeted diseases as a result of a disease prioritization workshop.

The objectives of this paper are to: 1) present animal health data collected from 1996 through the end of 2005, 2) review the progress made in the development of the national veterinary infrastructure through the cooperation established with international partners, and 3) discuss proposed future activities based on the experience gained.

Development of surveillance infrastructure

Animal disease reporting system in Bosnia and Herzegovina

The livestock population in BiH was substantially reduced as a consequence of the devastating war that occurred in the early 90's. As reported previously (5), there was a decline in the animal population by more than 60% in a large part of the country. Unfortunately, exact population data still do not exist, and available studies provide conflicting figures. A cattle identification and movement control scheme has been in existence since 2003, however, estimates are that only about 50 % of the cattle population has been registered (6). According to current estimates BiH has approximately 400,000 cattle, 1.000,000 sheep and goats, 400,000–600,000 pigs, 17,000-31,000 horses and 3.000,000 poultry (5).

Animal health care is primarily provided by a network of public and private veterinary practices. Disease surveillance is accomplished through a complex arrangement, as laboratory samples are submitted by multiple sources, including veterinary practices, producers, and veterinary inspectors. Laboratory diagnosis is organized through a network of eight animal health diagnostic labs. However, all of these labs essentially provide identical levels of services. There is a need to develop specialization within the laboratory system, to facilitate the development of expertise in the individual labs.

During the period 1995 to 2003, the disease reporting system was organized exclusively at the entity level and data were compiled from various independent sources. Data were collected through a passive system and rarely exchanged between agencies and stakeholders (7). Diseases were reported by sources from the entity veterinary sectors and included leptospirosis, Q fever, rabies, brucellosis of sheep, trichinellosis and Classical Swine Fever (CSF). A centralized disease reporting system was introduced in May, 2003 and improvements were made in the collection of data from both the entity administrations and through laboratory sources (Figure 1).

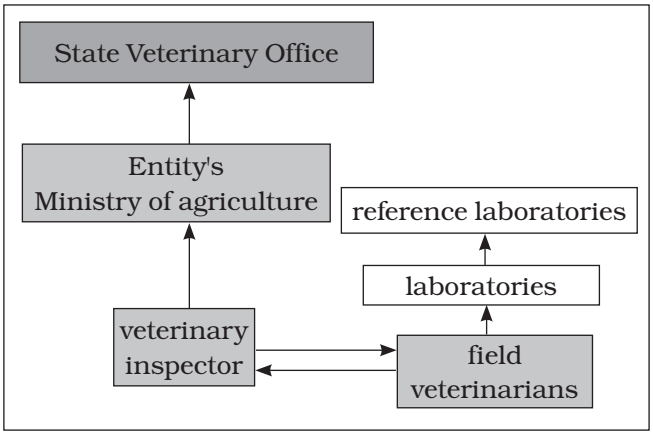

Figure 1: Scheme of sample submission and data flow within current disease reporting system in BiH

Targeted actions on improvements in the reporting of diseases such as brucellosis, bovine leptospirosis and CSF illustrated the importance of developing more comprehensive and scientifically based surveillance. However, the previous lack of scientific approaches to disease surveillance and current undetected disease occurrence has escalated the number of cases of sheep brucellosis and, consequently, the number of human brucellosis cases. Currently available data indicate rabies, CSF, Q fever and brucellosis may be endemic in the country (Figure 2).

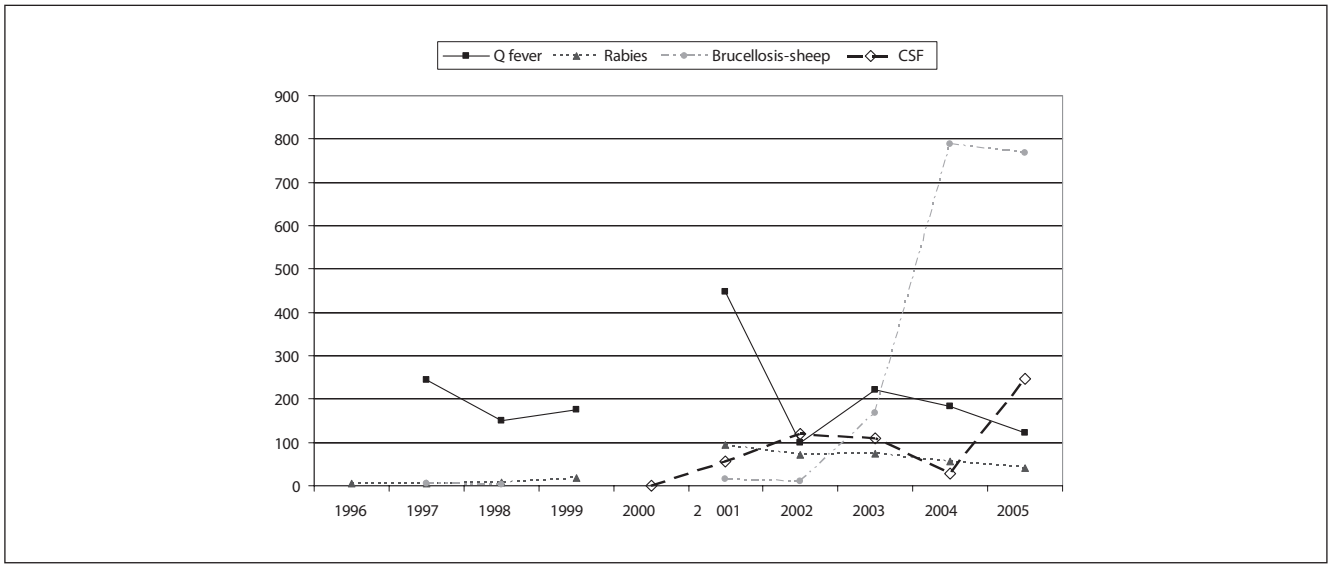

Figure 2: Reported outbreaks* of several important diseases (q fever, small ruminant brucellosis, rabies and classical swine fever) in BiH from 1996 to 2005

*definition of an outbreak adopted from the current legislation on reporting of the occurrence of animal diseases whereas the outbreak constitutes occurrence of one or more disease cases in the same epidemiological unit (most commonly farm, or backyard)

The increase in sheep brucellosis cases has many possible but no scientifically plausible explanations, as none of the disease or animal population at risk data were collected actively during the reported period.

Steps taken and progress made though international cooperation

International efforts were initiated in 2000 to assist the country in developing capacity in its animal health infrastructure. It was clear that due to its lack of domestic resources, the BiH veterinary community needed to acquire outside knowledge on the epidemiology of infectious diseases and surveillance strategies. An initial mission was conducted in BiH by a team from the APHI of Colorado State University in spring 2001. A plan was then created to address institutional development and approaches for solving significant animal health issues. This plan was presented to all levels of government and to all stakeholders. It placed emphasis on the initiation of educational and training activities and it made strong recommendations for the establishment of a central veterinary body along with the development of coordination plans between veterinary agencies on different administrative levels within the country. In accordance with this plan activities were conducted during the period from 2001 to 2005:

- In September 2001 a joint USDA-APHIS: Centers for Epidemiology and Animal Health (CEAH) and Colorado State University (CSU): APHI seminar was conducted in Sarajevo on animal health control programs;
- A workshop on animal disease surveillance was held in Sarajevo in September 2003. This was also a joint USDA-APHIS:CEAH and CSU:APHI project;
- A workshop was conducted on animal disease control prioritization in Sarajevo in October 2004;
- A workshop with title “Food safety solutions: status and perspectives in Bosnia and Herzegovina”, was conducted in Sarajevo in May, 2005;

- Two young professionals from the Veterinary faculty Sarajevo successfully completed a master's degree in preventive veterinary medicine (MPVM) and a Master of Science degree at the University of California at Davis and CSU, respectively.

Results

International efforts delivered within collaboration of above listed scientific institutions from USA and BiH had catalyzed development in the animal health infrastructure and assisted in achievement of several important objectives during reported period:

- A functioning State Veterinary Administration was established under the Ministry of Foreign Trade and Economic Relations (MFTER) in December 2000.
- A state veterinary law was adopted in October, 2002.
- An Animal health economics center was established in 2003 as joint effort of the Veterinary faculty Sarajevo, Animal Health Population Institute of Colorado State University and USDA:APHIS.
- Compilation and distribution of monthly epidemiology reports to domestic and international veterinary authorities and trading partners began in May 2003.
- In fall 2004 an agreement was made with neighboring countries to create a regional task force to develop a regional animal disease surveillance program.
- A surveillance project to detect current levels of brucellosis in sheep was conducted by a member of the Veterinary faculty in 2006 as part of a master's degree program in veterinary epidemiology. During this project it was determined that "official" figures for brucellosis prevalence are far below the true levels. A direct result of this project will be to improve the control of brucellosis in animals and humans.

As a result of the disease prioritization exercise for significant animal diseases conducted during October 2004 disease prioritization matrix was created (Table 1). Prioritization working group was made from representatives of veterinary and public health administration from BiH and neighboring countries (Croatia and Serbia) and animal producers from BiH. The group first selected factors they considered to be significant in prioritizing disease surveillance activities (public health impact, interest to region, impact to animal production, impact on export, etc.). Once this was accomplished, each evaluating factor was ranked according to its relative importance (with five being the most important factor). A discussion was held to determine which diseases would be evaluated by this system. Rabies, brucellosis, Q fever, tuberculosis, CSF, bovine spongiform encephalopathy and bluetongue were selected. The group then weighted consequences of selected diseases on every factor individually using scale of one to three (with three being the most significant consequences). Once the ranking of each disease was established in accordance with each of the evaluating factor, priorities were determined. Score for a disease was tabulated by taking its rank for each factor affection priority level of a disease (column) and multiplying it by the weighting factor for the respective consequences (raw). These scores were then totaled for each disease across all prioritization factors (columns). These totals determined the individual disease ranking.

Final evaluation of these diseases considered other factors such as costs and the feasibility of conducting surveillance on multiple disease agents within a single surveillance system. Using prioritization matrix, brucellosis (overall score 50) and bovine spongiform encephalopathy (overall score 50) were identified as the most important diseases, followed by tuberculosis (48), Q fever (45), rabies (43), classical swine fever (41) and bluetongue (26).

Current and future challenges

Work must progress towards accession to the WTO, and this will require even more dedicated commitment from the veterinary services in the country. As well, work must continue towards harmonization with the EU economic space, another project that will require a significant contribution from the veterinary sector. A major goal will be to reassess the current disease information system and adjust it to prevailing surveillance requirements. The resulting system must involve participation of all agencies within the veterinary services of BiH. This project will include the design and implementation of monitoring and surveillance plans for important infectious animal diseases.

The establishment of scientifically based surveillance for diseases identified as the top priority will result in reliable, complete and timely acquired data and will be a key factor for gaining entry into the WTO and harmonizing with the European Commu-

Table 1: Disease prioritization matrix for Bosnia and Herzegovina

Ranking/ weight of consequences		Public health	Interest to region	Impact on production	Impact on export		Feasibility of control	Cost of control	Public opinion
					Animals	Products			
		5	3	3	1	2	2	4	2
High	3	*R, B, Q	TB	CSF	CSF, B, BSE, BT	CSF, BSE	TB	R, BSE, Q	R, TB, BSE, Q
Medium	2	TB, BSE	R, CSF, B, BSE, BT	B, TB	Q	B	R, B, BSE	CSF, B, TB, BT	B
Low	1		Q	BT, BSE, Q	TB	TB, BT, Q	CSF, BT, Q		CSF, BT
No	0	CSF, BT		R	R	R			

Legend: *- R– rabies, B– brucellosis, Q– Q fever, CSF– Classical Swine Fewer, TB– tuberculosis, BSE– Bovine Spongiform Encephalopathy, BT– Bluetongue

nity. In contrast to what are current practices and what is prescribed by outdated legal requirements, future animal disease surveillance activities should rely on systematically collected data from field. The key elements for this include; defining cost-efficient and statistically justified sample size and frequency of samples collection, computerization of data management and clearly defined format and addresses for disease reports distribution. A related goal will be to develop future coordination of activities between animal health and public health agencies. As mentioned earlier, an agreement has been reached with neighboring countries to create and implement a regional task force to develop an animal health reporting system.

Conclusion

It is evident that transitional and developing countries face great challenges in meeting international requirements for animal health, public health and animal welfare. However, international cooperation and support focusing on the transfer of knowledge on epidemiology and surveillance activities could be very helpful in meeting this goal. Our work demonstrated that the improvement of the animal disease reporting system is requisite for the establishment of a surveillance infrastructure. Future activities might be focused on more specific training for surveillance and control of diseases identified as national priorities, improvement of diagnostic capabilities and the identification of appropriate funding for surveillance activities.

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RAZVOJ NADZORNEGA SISTEMA ZA OHRANJANJE ZDRAVJA ŽIVALI V BOSNI IN HERCEGOVINI – STROKOVNO POROČILO

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Povzetek: Bosna in Hercegovina se od leta 1996 intenzivno ukvarja s postavitvijo nacionalne veterinarske infrastrukture, da bi izboljšala zdravstveno stanje živali, zdravje ljudi in varnost prehrane. Številne mednarodne agencije in donatorji so namenili precejšnja sredstva za obnovitev živinoreje, ki je bila hudo prizadeta med zadnjo vojno (od aprila 1992 do novembra 1995). Precejšen trud je bil vložen tudi v izboljšanje veterinarske službe v državi. Izboljšave so postale očitne, ko je bila decembra 2000 ustanovljena državna veterinarska uprava. Nedavna pogajanja o priključitvi k Svetovni trgovinski organizaciji (WTO) in prizadevanja, da bi ustrezali sanitarnim in fitosanitarnim sporazumom, so pokazala potrebo po nadaljnji analizi zdravstvenega stanja in nevarnih bolezni na državnem nivoju.
Center za ekonomiko zdravja živali pri Veterinarski fakulteti v Sarajevu od leta 2001 sodeluje z Inštitutom za zdravje živalskih populacij iz Kolorada (Animal Population Health Institute, Colorado State University). V tem času poteka več dejavnosti za razvoj in ustanovitev učinkovite nacionalne veterinarske infrastrukture. Ministrstvo za kmetijstvo iz ZDA (Department of Agriculture) je podprlo učne programe in tehnične delavnice. Do danes so z izobraževanjem pokrili naslednja področja: programe za preverjanje zdravja živali, nadzorstvo, državne prednostne liste bolezni, varnost prehrane in spodbujanje regionalnega sodelovanja. Sedaj se razvijajo in uvajajo državni nadzorni sistemi za bolezni, ki so bile izbrane na delavnicah za prednostne liste bolezni.

Ključne besede: Bosna in Hercegovina; veterinarska služba – razvoj

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Article in a journal or newspaper: Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

Article in proceedings of a meeting or symposium: Schnoebelen CS, Louveau I, Bonneau M. Developmental pattern of GH receptor in pig skeletal muscle. In: the 6th Zavrnik memorial meeting. Lipica: Veterinary Faculty 1995: 83-6.

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Načini citiranja

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