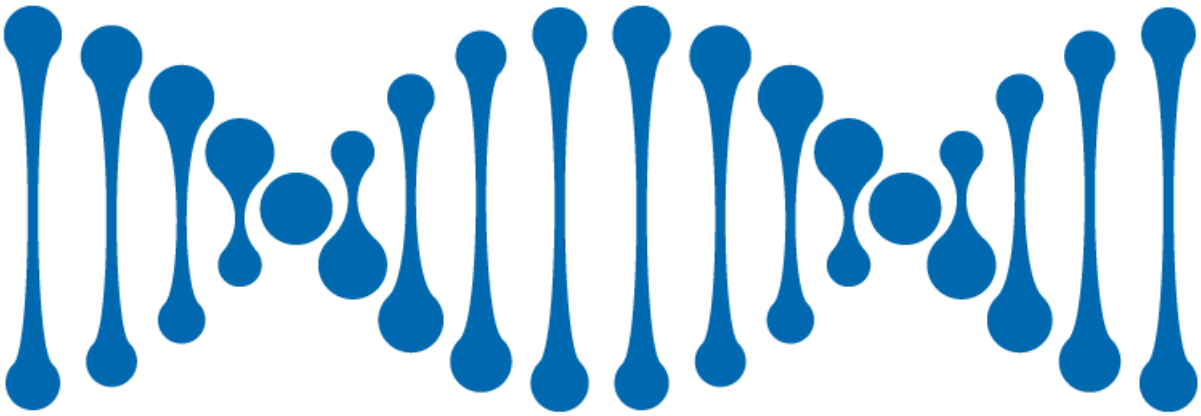
GENETIC SOCIETY OF SLOVENIA

IN COLLABORATION WITH

THE SLOVENIAN SOCIETY OF HUMAN GENETICS

PROCEEDINGS

6th COLLOQUIUM OF GENETICS



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OPENING LECTURE

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CRISPR/CAS9 DELIVERY BY EXTRACELLULAR VESICLES FOR GENOME EDITING AND THERAPEUTIC TRANSCRIPTIONAL REGULATION

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POVZETEK

Sistem CRISPR/Cas predstavlja pomembno orodje za modifikacijo genoma in regulacijo izražanja genov. Omenjen sistem sestavlja endonukleaza Cas9 ter kompleks RNA, ki vodi protein Cas9 na izbrano tarčno regijo v genomu. Z uporabo CRISPR/Cas9 lahko ustvarjamo organizme z izbitim genom ali organizme z vstavljenim zaporedjem tuje DNA. Druga močna uporabnost omenjenega sistema je vplivanje na izražanje genov. Z uporabo katalitsko neaktivnega proteina Cas9, povezanega z aktivacijskimi ali represorskimi domenami, lahko vplivamo na povečano ali zmanjšano izražanje posameznih genov. Uporabnost sistema CRISPR je praktično neomejena glede na tip organizma, vendar pa obstajajo še vedno velike omejitve vnosa sistema *in vivo* za morebitne terapevtske učinke. Zunajcelični vezikli so sposobni dostavljati raznolike molekule. Z našim delom smo pokazali učinkovito pakiranje in dostavo sistema CRISPR/Cas9 v zunajcelične vezikle. Z zunajceličnimi vezikli smo uspešno dostavili endonukleazo Cas9 kot tudi umetni transkripcijski faktor na osnovi dCas9-VPR, kar je omogočilo modifikacijo genoma ter vplivanje na povišano izražanje izbranih tarčnih genov. Funkcionalno dostavo sistema CRISPR/Cas9 z uporabo zunajceličnih veziklov smo pokazali na različnih celicah celičnih linij, primarnih celicah ter tudi v živalih. Z dostavo dCas9-VPR in sgRNA kompleksa preko zunajceličnih veziklov smo pokazali terapevtsko učinkovitost na mišjem modelu hepatotoksičnosti.

Ključne besede: sistem CRSIPR/Cas system, zunajcelični vezikli, modifikacija genoma

ABSTRACT

The CRISPR/Cas system emerged as the highly potent tool for genome engineering and regulation of gene transcription. This novel gene editing tool consists of Cas9 enzyme, representing the scissors and RNA complex as the precise targeting component. Using CRISPR/Cas9 system we can make knockout models by introducing indel mutations or knock in models by codelivery of a donor DNA that reacts like a DNA template for the repair. Another important benefit of CRISPR/Cas system is highly efficient gene expression alteration. By using catalytically inactive endonuclease dCas9 that possesses no activity and acts only as a binding tool to DNA to recruit heterologous activation or

repression domains we can regulate gene expression. One of the additional important features of CRISPR is that it can function in basically any type of cells or organism. However the efficiency of its delivery into cells, particularly for safe therapeutic *in vivo* applications, remains a major bottleneck. Extracellular vesicles, released by cells, can mediate the transfer of different functional molecules. We have shown the efficient packaging and delivery of the CRISPR/Cas system via extracellular vesicles to target cells. Extracellular vesicles can transfer the functional Cas9 or designed transcriptional regulator dCas9-VPR, enabling genome editing or regulating gene transcription. Delivery and robust genome editing and gene upregulation function was shown for cell lines, primary cells and in the animals. *In vivo* delivery of dCas9-VPR/sgRNA by using extracellular vesicles demonstrated therapeutic efficiency in a mouse model of liver damage, which opens the path towards therapeutic applications.

Key words: CRSIPR/Cas system, extracellular vesicles, genome editing

SHORT LECTURES – BIOTECHNOLOGY

Ester Stajič

PROTOCOL DEVELOPMENT FOR GENOME EDITING IN CABBAGE (*Brassica oleracea* VAR. *capitata L.*) USING CRISPR/CAS9 TECHNOLOGY

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PROTOCOL DEVELOPMENT FOR GENOME EDITING IN CABBAGE (*BRASSICA OLERACEA* VAR. *CAPITATA* L.) USING CRISPR/CAS9 TECHNOLOGY

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POVZETEK

Tehnologija CRISPR/Cas9 je zaradi enostavnosti oblikovanja trenutno najbolj priljubljena metoda za vnašanje specifičnih mutacij v genom. Protokoli za tarčno ciljanje genomov s sistemom CRISPR/Cas9 so bili objavljeni za številne organizme, pri zelju pa takšnega protokola še ni. Zato je naš namen vzpostaviti protokol za tarčno mutagenezo s tehnologijo CRISPR/Cas9 pri zelju. Za ta namen bomo tarčno spreminjali centromerni protein CENH3, ki ima pomembno vlogo pri ločevanju kromatid med mitozo in mejozo. Pred kratkim objavljene točkovne mutacije v proteinu CENH3 so po opraševanju s takšno rastlino pri vrsti *Arabidopsis thaliana* vodile v nastanek haploidnega potomstva, kar nakazuje na možnost uporabe rastlin s takšnimi mutacijami za indukcijo haploidov. V naših poskusih bomo za induciranje mutacij pri proteinu CENH3 uporabili dva pristopa: stabilno transformacijo somatskih celic in prehodno izražanje plazmidov v protoplastih. Zato smo najprej vzpostavili protokol za stabilno transformacijo ter protokol za izolacijo protoplastov. Za uspešno regeneracijo protoplastov smo preverili vpliv različnih rastnih pogojev, kot je gojenje v temi ali na svetlobi in različna gojišča za regeneracijo kalusa. Prav tako smo izdelane ekspresijske vektorje vnesli s transformacijo hipokotilov z *Agrobacterium tumefaciens* in trenutno preverjamo prisotnost želenih mutacij pri regeneriranih rastlinah.

Ključne besede: zelje, tarčna mutageneza, CRISPR/Cas9, CENH3

ABSTRACT

CRISPR/Cas9 technology is due to its simplicity for design currently a method of choice for introducing specific genetic modifications. Protocols for genome editing using CRISPR/Cas9 system have been published for many different organisms, but so far there is no protocol for targeted mutagenesis in cabbage. Therefore, our aim is to develop a protocol for genome editing in cabbage using CRISPR/Cas9 system. For that purpose, we chose to target the centromere protein CENH3, which has an important role in segregation of chromatids during mitosis and meiosis. Recently published point mutations in CENH3 protein resulted in haploid offspring in *Arabidopsis thaliana* suggesting that plants carrying those mutations could be used as haploid inducers. In our experiments, mutations in CENH3 will be induced through two different approaches: stable transformation of somatic cells and transient expression of plasmids in protoplasts. We have previously developed a protocol for *Agrobacterium tumefaciens* mediated transformation and a protocol for protoplast isolation. For successful regeneration of protoplasts different growth conditions, such as incubation in dark or in light, and several different media for regeneration of calli were tested. We have also introduced the constructed expression vectors through transformation of

hypocotyls with *Agrobacterium tumefaciens* and we are currently in the process of screening of regenerated plants for desired mutations.

Key words: cabbage, genome editing, CRISPR/Cas9, CENH3

SHORT LECTURES – MOLECULAR BASIS OF DISEASES

Helena Volk

CHARACTERISATION OF NOVEL *Verticillium nonalfalfae* CHITIN BINDING EFFECTOR

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PERITUMORAL GENE ELECTROTRANSFER OF IL-12 AS ADJUVANT THERAPY TO PLATINUM-BASED ELECTROCHEMOTHERAPY FOR TREATMENT OF MURINE MALIGNANT MELANOMA

Monika Savarin

VASCULAR TARGETED GENE ELECTROTRANSFER RADIOSENSIBILAZES TUMORS

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CHARACTERISATION OF NOVEL *Verticillium nonalfalfae* CHITIN BINDING EFFECTOR

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POVZETEK

Identifikacija tarčnih proteinov gostitelja je eden izmed najpomembnejših vidikov pri preučevanju biologije efektorjev in pri načrtovanju odpornosti na bolezni. *Verticillium nonalfalfae* izloča *Vna*CBP8.213, ki se močno izraža predvsem v občutljivi sorti hmelja. Je 38.6 kDa velik efektorski protein z 12,5 % cisteina in s šestimi predvidenimi domenami heveina (CBM18), ki vežejo ogljikove hidrate. Vlogo *Vna*CBP8.213 smo preučili z lokalizacijskimi študijami, kvasnim dvohibridnim sistemom in z rekombinantnim proteinom v biokemijskih testih. *Vna*CBP8.213 izražen kot fuzijski protein z mRFP v *Nicotiana bentahmiana* smo odkrili v citoplazmi in jedru, z nekoliko manjšo prisotnostjo v jedrcu. V knjižnici cDNA klonov iz krompirja smo s kvasnim dvohibridnim sistemom identificirali zgolj tri šibke interakcije med *Vna*CBP8.213 in proteini krompirja. Pravilno zvitje in toplotno stabilnost rekombinantnega *Vna*CBP8.213, pridobljenega iz inkluzijskih telesc *E. coli* SHuffle celic, smo preverili s cirkularnim dikroizmom. Vidnega preobčutljivostnega odgovora v listih *Nicotiana bentahmiana* nismo odkrili niti po infiltraciji rekombinantnega proteina niti po izražanju *in planta*. S testom sedimentacije z ogljikovimi hidrati smo potrdili vezavo rekombinantnega proteina na hitinske kroglice, hitin iz lupin rakov, do vezave pa ni prišlo na celulozo in ksilan. Pokazali smo, da *Vna*CBP8.213 rastlinski imunski sistem ne prepoznava kot PAMP (s patogeni povezan molekulski vzorec). Predpostavljamo, da *Vna*CBP8.213 omogoči *Verticillium nonalfalfae*, da se izmuzne rastlinskemu imunskemu odzivu tako, da veže proste hitinske oligomere ali da zaščiti glivno celično steno pred razgradnjo z rastlinskimi hitinazami.

Ključne besede: CBM18, vezava hitina, efektor, *Verticillium nonalfalfae*, hmelj
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ABSTRACT

One of the most important aspects of studying effector biology is the identification of host targets that could be exploited in engineering disease resistance. *Vna*CBP8.213 is a *Verticillium nonalfalfae* secreted effector protein, which shows high gene expression in a susceptible hop cultivar. The 38.6 kDa protein contains 12.5 % cysteines and has six predicted carbohydrate binding protein hevein (CBM18) domains. The function of *Vna*CBP8.213 was assessed here by sub-cellular localization studies, yeast-two-hybrid screening and biochemical tests using recombinant protein. *Vna*CBP8.213, expressed as mRFP fusion protein in *Nicotiana bentahmiana*, localized to cytoplasm and nucleus, with some exclusion from the nucleolus. Yeast-two-hybrid screening of the available potato cDNA library indicated only three weak interactions between *Vna*CBP8.213 and potato proteins. *Vna*CBP8.213 was expressed in *E. coli* SHuffle cells and re-solubilized from inclusion bodies. The correct folding and thermal stability of the recombinant protein was confirmed by circular

dichroism. The infiltrated recombinant protein and the *in-planta* expressed protein did not trigger a visible HR response in *Nicotiana benthamiana* leaves. Carbohydrate sedimentation assay confirmed recombinant protein binding to chitin beads, crab shell chitin but not to cellulose and xylan. We demonstrated that *Vna*CBP8.213 is not recognized by the plant immune system as a PAMP (pathogen-associated molecular pattern). We hypothesize it is involved in the evasion of cell wall triggered immune responses, either as a scavenger of chitin oligomers or by protecting the fungal cell wall from degradation by plant chitinases.

Key words: CBM18, chitin binding, effector, *Verticillium nonalfalfae*, hops
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ABERRANT EPIGENETIC NETWORK OF lncRNAS AND mRNAS IN GLIOMA DEVELOPMENT AND PROGRESSION

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POVZETEK

Dolge ne-kodirajoče RNA (lncRNAs) so ene izmed epigenetskih mehanizmov, vključenih v nastanek in nadaljnji razvoj različnih tumorjev, tudi gliomov. lncRNA kontrolirajo izražanje protein-kodirajočih genov na različnih nivojih njihovega prepisovanja in genov, ki so vključeni v različne signalne poti. Spremembe v izražanju so dokazano povezane s specifičnim tipom tumorja, zato smo želeli določiti profil izražanja genov pri gliomih in poiskati nove kandidatne (ne)kodirajoče gene, vključene v nastanek gliomov. Gliomi so klinično izredno heterogeni primarni možganski tumorji, ki kljub napredkom v molekularni biologiji predstavljajo problem za zdravljenje. Njihova heterogenost je opazna v pomanjkljivem znanju o mehanizmih, ki vodijo v nastanek in napredovanje tumorja. Primerjalne analize izražanja genov v vzorcih gliomov so pokazale, da jih lahko razvrstimo v podskupine glede na podobnosti v profilu izražanja. Tako smo z uporabo mikromrež na manjšem številu biopsij gliomov določili profil izražanja 879 lncRNA in 477 potencialnih tarčnih mRNA, ki so vključene v epigenetske signalne poti. Številni tako ne-kodirajoči kot kodirajoči geni so pokazali statistično značilne spremembe v njihovem vzorcu izražanja. Večina tarčnih mRNA je vključenih v epigenetske procese kot so (de)metilacija ali modifikacije histonov. Rezultati izražanja so bili filtrirani in preverjeni na selekcioniranih lncRNA/mRNA, ki so bile nadalje vključene v analizo določanja metilacijskega statusa promotorja. V različnih histopatološko-določenih tipih gliomov smo našli izrazite spremembe tako v vzorcu izražanja kot v vzorcu metilacije posameznih lncRNA/mRNA, ki bi jih lahko potencialno uporabili kot dodatne diagnostične označevalce pri sami klasifikaciji gliomov. Nedvoumno je, da spremembe v izražanju lncRNA, podobno kot mRNA, znatno prispevajo k razvoju gliomov in napredovanju bolezni.

Ključne besede: lncRNA, epigenetika, gliomi, profil izražanja, metilacija, biooznačevalci

ABSTRACT

lncRNAs are one of the key epigenetic mechanisms known to be involved in development and progression of different tumours, including glioma. These RNAs are regulators of crucial protein-coding genes on multiple levels and various signalling pathways. Aberrant gene expression is already shown to be in correlation with specific tumour type and our purpose was to screen glioma expression profile for novel potential (non)coding biomarkers of glioma development. Glioma are clinically extremely heterogeneous primary brain tumours that are presenting a great challenge for treatment in clinical oncology. Their heterogeneity is reflected in the lack of knowledge about the exact mechanisms leading to tumour formation and progression. Comparative studies of gene expression showed that we can arrange glioma in different subtypes upon similar expression

patterns. Using the microarray technology, we screened a smaller cohort of fresh biopsy tissue samples for expressional differences of 879 lncRNAs and 477 potential mRNA targets, which are related to epigenetic signalling pathways. We determined a significant change in expression of numerous non-coding as well as coding genes, crucially involved in epigenetic processes such as (de)methylation or histone modification. Microarray results were filtered and several lncRNAs/mRNAs were included in expression validation and further methylation analysis. We found a distinctive gene's expression and methylation patterns in different histological subtypes, which may contribute an additional diagnostic value in glioma classification. Moreover, changes in expression and promoter methylation also show potential prognostic value. Unequivocally, aberrantly expressed lncRNAs, like mRNAs, are an important component in glioma development and progression.

Key words: lncRNAs, epigenetics, glioma, expression profile, methylation, biomarkers

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PERITUMORAL GENE ELECTROTRANSFER OF IL-12 AS ADJUVANT THERAPY TO PLATINUM-BASED ELECTROCHEMOTHERAPY FOR TREATMENT OF MURINE MALIGNANT MELANOMA

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POVZETEK

Kombiniranje že uveljavljenih terapij z nedavno odkritimi imunoterapijami je obetajoče za zdravljenje malignega melanoma. Predmet naše raziskave je intratumoralna elektrokemoterapija (i.t. EKT) kot lokalna ablativna terapija v kombinaciji s peritumoralnim genskim elektroprenosom (p.t. GEP; plazmidna DNA in elektroporacija) interlevkina-12 (IL-12) kot adjuvantno terapijo. Medtem ko je EKT s cisplatinom (CDDP) že dobro uveljavljena v kliniki, EKT z oksaliplatinom (OXA) do danes še ni bila raziskana. OXA je kandidat za EKT na podlagi podobnosti s CDDP in zaradi predpostavljeno večjega imunomodulatornega vpliva. Na melanomskem modelu smo pokazali, da je EKT z OXA enako učinkovita kot EKT s CDDP, če dozo OXA dvignemo za faktor 1,6. Izpostavitev melanomskih celic EKT vodi v imunogeno celično smrt, ob uporabi CDDP ali OXA, kar kolerira s primerljivo infiltracijo imunskih celic v tumor. Kljub temu smo z EKT s CDDP ali OXA opazili le zaostanek v rasti primarnih tumorjev, ne pa tudi popolnih odgovorov. Z namenom doseganja boljše lokalne tumorske kontrole in tudi protitumorskega učinka na oddaljene nezdravljene metastaze, smo terapiji dodali p.t. GEP plazmida z zapisom za IL-12. Samostojna terapija GEP IL-12 je povzročila le zaostanek v rasti tumorjev, medtem ko smo s kombinirano terapijo (EKT s CDDP in GEP IL-12) dosegli 30 % popolnih ozdravitev ter tudi sistemski učinek na oddaljene tumorje. Protitumorski učinek kombinacije EKT z OXA in IL-12 je bil manjši. Rezultati raziskave so pokazali, da GEP IL-12 potencira učinek EKT na lokalni in sistemski ravni.

Ključne besede: elektroporacija, cisplatin, oksaliplatin, interlevkin-12

ABSTRACT

Combining established therapies with recently discovered immunotherapies is promising for treatment of malignant melanoma. We have been studying intratumoral electrochemotherapy (i.t. ECT) as a local ablative therapy in combination with peritumoral gene electrotransfer (p.t. GET; plasmid DNA and electroporation) of interleukin-12 (IL-12) as adjuvant therapy. While ECT with cisplatin (CDDP) is already a well-established treatment in clinics, oxaliplatin (OXA) has not been tested for use with ECT yet. Based on the similar characteristics between OXA and CDDP and the presumably more pronounced immunomodulatory effects of OXA, OXA is a candidate for ECT. We showed that ECT with OXA is as effective as ECT with CDDP in melanoma model when the OXA dose is increased 1.6-fold. Exposure of melanoma cells to ECT induced immunogenic cell death with both drugs, which correlated with a comparable increase in lymphocyte infiltration into tumors. However,

with ECT alone we observed only a delayed growth of transplanted tumors with no complete responses. With the aim to achieve both better local tumor growth control and an effect on distant non-treated metastasis (abscopal effect), we applied p.t. GET of plasmid encoding for IL-12. Treatment with IL-12 GET alone resulted only in tumor growth delay, whereas with combined therapy (ECT with CDDP and GET with IL-12) we cured 30% of animals and also abscopal effect was observed. Antitumor effectiveness of combined ECT with OXA and GET with IL-12 was less pronounced. Our findings indicate that GET with IL-12 potentiates the effect of ECT on local and systemic level.

Key words: electroporation, cisplatin, oxaliplatin, interleukin-12

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VASCULAR TARGETED GENE ELECTROTRANSFER RADIOSENSIBILAZES TUMORS

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POVZETEK

Tumorji večji od 2 mm^3 potrebujejo lastno žilje za nadaljnjo rast, razvoj in metastaziranje. Tumorsko žilje je ključnega pomena pri klasičnih terapijah, kot je obsevanje, kot tudi pri novejših, žilno ciljanih. Pri slednjih je bistven izbor specifične tarče za proliferativno tumorsko žilje. Ena izmed teh je endoglin, ki je kot koreceptor TGF-β, udeležen v kompleksni signalni poti endotelijskih celic. Dokazali so, da utišanje njegovega izražanje bistveno vpliva na zaostanek v rasti tumorjev, a je bila taka terapija zaradi uporabe kratkoživih ali manj specifičnih molekul (protitelesa, siRNA) kratkotrajna. Da bi podaljšali čas delovanja terapije, smo razvili plazmid, ki kodira shRNA proti endoglinu in ima promotor specifičen za endotelijske celice. V celice in tkiva smo ga vnašali s pomočjo genskega elektroprenosa, ter preverjali njegovo specifičnost in učinkovitost. *In vitro* smo najprej potrdili, da imamo tkivno specifičen plazmid, ki ima žilno ciljano delovanje. *In vivo* smo njegovo učinkovitost preverili na dveh tumorskih modelih, ki se razlikujeta v izražanju endoglina. Ugotovili smo, da ima tak genski elektroprenos bistven žilno ciljani učinek, ki prispeva k značilnemu zaostanku v rasti tumorjev. Poleg tega, smo zaradi normalizacije žilja dosegli značilno radiosenzibilizacijo tumorjev, kar je prispevalo k visokemu deležu popolnih ozdravitev miši. Na melanomskem tumorskem modelu smo ugotovili, da s kombinacijo genskega elektroprenosa in obsevanja spodbudimo delovanje imunskega sistema, ki je prispeval k skoraj 90% deležu popolnih ozdravitev, ter k vzpostavitvi dolgotrajnega imunskega sistema. Trdimo lahko, da smo razvili terapijo, ki ima prihodnost tudi v nadaljnjih kliničnih študijah, predvsem pri tumorjih neodzivnih na VEGF signalno pot.

Ključne besede: genski elektroprenos, plazmid, žilno tarčna terapija

ABSTRACT

Tumors, bigger than 2mm^3, need their own vasculature for development, growth and metastasis. Tumor vasculature is a key player in standard treatment modalities, as radiotherapy, as well as in new, vascular targeted therapies. In the later, specific target is important. One of the potential targets is endoglin, a co-receptor of TGF-β, involved in complex signaling pathway of endothelial cells. Silencing of endoglin expression has resulted in good tumor growth delay, although the effectiveness was not long-lasting due to the usage of unstable molecules, like antibodies and siRNA. Therefore, the aim of our study was to construct plasmid encoding shRNA against endoglin and having specific promoter for endothelial cell. The plasmid was introduced into the cells and tissue by using gene electrotransfer, and thereafter the specificity and effectiveness were evaluated. *In vitro*, we have demonstrated to have tissue specific plasmid with vascular targeted effectiveness

that was further evaluated in *in vivo* studies, were two tumor models, with different status of endoglin expression, were used. We demonstrated that in both tumor models such gene electrotransfer results in vascular targeted effectiveness and significant tumor growth delay. Due to the vessel normalization, we were able to achieve significant radiosensibilization of tumors and 50% of complete responders. In melanoma tumor model, the combination of both treatment modalities resulted in activation of an immune system, which led to 90% of complete responders, and formation of immune memory. This therapeutic approach has a promising future in clinics, especially for a tumors refractory to VEGF signaling pathway.

Key words: gene electrotransfer, plasmid, vascular targeted therapy

SHORT LECTURES – GENOMICS

Jana Obšteter

THE USE OF GENOMIC INFORMATION IN CATTLE BREEDING

Erik Rihtar

HORIZONTALY ACQUIRED PATHOGENICITY ISLAND PAI*usp* OF *Salmonella bongori* AND UROPATHOGENIC *Escherichia coli*, IS A NEW MEMBER OF THE TyrR REGULON

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THE USE OF GENOMIC INFORMATION IN CATTLE BREEDING

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IZVLEČEK

Selekcija stremi k genetskemu izboljšanju populacije. Klasična selekcija pri govedu je odvisna predvsem od lastnega fenotipa in fenotipov potomcev, kar se odraža v dolgem generacijskem intervalu. Uspeh selekcije v celotnem rejskem programu je odvisem od intenzivnosti in točnosti selekcije ter generacijskega intervala. Vsi ti parametri se pri govedu bistveno razlikujejo med moško in žensko potjo selekcije. Vse večja dostopnost genomske informacije sedaj omogoča bolj točno napoved plemenskih vrednosti mladih nefenotipiziranih živali, kar skrajša generacijski interval. Namen te raziskave je bil z uporabo stohastične simulacije testirati različne genomske scenarije na realistični populaciji govedi. Za ta namen je bil razvit holističen simulator, ki vključuje stohastično generiranje živali in genomov, napoved plemenskih vrednosti, selekcijo in parjenje. Selekcijski parametri za simulacijo so bili povzeti po slovenski populaciji rjavega goveda. Testirani scenariji so vključevali klasičeni in štiri genomske scenarije. Slednji so se razlikovali v uporabi genomske informacije. Klasični scenarij je imel najmanjši genetski napredek (0.10 SD), genomski scenarij z uporabo genomske informacije v obeh moških poteh selekcije pa je imel največji genetski napredek (0.18 SD). V splošnem so imeli scenariji z večjo redukcijo generacijskega intervala tudi večji uspeh.

Ključne besede: govedo, strategije selekcije, simulacija, genomska informacija

ABSTRACT

Selection aims for genetic improvement of a population. Conventional selection in cattle is highly dependent on own and progeny phenotypes and consequently characterised with long generation intervals. The success of selection in the whole breeding program is influenced by intensity, accuracy of selection and generation interval, which differ substantially between the male and female selection paths in cattle breeding. Nowadays, the increasing availability of genomic information enables more accurate estimation of breeding values for young non-phenotyped animals which reduces the generation interval. The aim of this research was to test different genomic scenarios in a realistic cattle population using stochastic simulation. A holistic simulator implementing stochastic generation of the animals and genomes, breeding value estimation, selection and mating was developed. The selection parameters were set to mirror the Slovenian Brown Swiss population. The tested scenarios included the conventional and four genomic scenarios differing in the use of genomic information. The conventional scenario resulted in the lowest genetic gain (0.10 SD) while the genomic scenarios using genomic information in both

male selection paths had the highest genetic gain (0.18 SD). In general, the success of the scenarios increased with decrease in generation interval.

Key words: cattle, breeding strategies, simulation, genomic information

INTRODUCTION

Selection in cattle aims for genetic improvement of a population. This includes screening economically important traits on many animals, inferring breeding values, and selecting the best individuals as parents of the next generation. Due to long generation interval, large impact of few selected males, and high costs of rearing the animals, selection decisions in cattle must be made with high confidence. However, the identification of genetically superior individuals is associated with large uncertainty. This is due to many factors: unknown genetic architecture for most economically important traits; substantial environmental effects; prevalent sex-limited expression of relevant traits; recombination and segregation of genomes from previously identified elite individuals; required interaction between farmers, their breeding organisations, and technical services such as milk recording, artificial insemination (AI), and genetic evaluation; and always present finite amount of financial resources to acquire sufficient amount of information that optimise return on investment.

Identification of superior breeding animals is tackled by statistical modelling of the collected data using the method of best linear unbiased predictions (BLUP) (Henderson, 1984). The model is used to estimate breeding value (EBV), which is twice the deviation of the progeny from the population mean (Falconer & Mackay, 1996). Traditionally, genetic evaluation utilises recorded pedigree relationship to combine phenotypic information of all animals. However, since young animals do not yet have own phenotypic information, their EBV is simply the average of their parents' EBVs (PA), which has low correlation with the true BV (accuracy) due to recombination and segregation of parental genomes. To achieve high accuracy, the breeder must wait for an animal to obtain at least their own phenotype. Sex-limited traits, such as milk production, pose additional challenge since males can be selected only after about 6 years when their daughters complete first lactation.

The sex-limited expression of phenotypes leads to a multi-stage selection in cattle. A generic cattle breeding program can be described in four steps: i) PA-based selection of male calves for own performance testing ii) EBV-based selection of best performance tested young males for progeny testing iii) EBV-based selection of best progeny tested males for wide-spread use in population iv) EBV-based selection of best females for insemination with elite males (contracted mating). Since the impact and contribution of selected males is much higher compared to females, a stronger selection is applied on the male path of selection. All these factors lead to different accuracies, selection intensities, and generation intervals in males and females and their corresponding male and female parents. Rendel and Robertson described these different ways to transmit the genes to the next generation as four "paths of selection": sires of sires, sires of dams, dams of sires and dams of dams (Rendel & Robertson, 1950).

The availability of reasonably low-cost single-nucleotide-polymorphism (SNP) arrays enabled the collection of genomic information and led to a revolution in cattle breeding, i.e. genomic selection (Meuwissen, Hayes, & Goddard, 2001). Genomic information allows estimation of (genomic) breeding value (gEBV) for all genotyped animals, even young non-phenotyped ones. While the accuracy of gEBV is generally lower than the accuracy of progeny test EBV, the ability to shorten

generation interval provides a large opportunity for cattle breeding. The genomic information is statistically modelled to replace or complement the pedigree information in genetic evaluation. Whereas a pedigree provides information about expected relationships, SNP genotypes provide information about realised relationships by capturing recombination and segregation of parental genomes.

Genetic gain depends on the intensity and accuracy of selection decisions, genetic variability, and generation interval with the latter being the average age of the parents at the birth of their selected offspring. Genomic selection enables a more accurate selection of young animals which reduces generation interval and therefore increases genetic gain. However, practical implementation is encompassed with a lot of technical difficulties, scepticism and precaution. Reasons for this are the uncertainty regarding the reduced generation interval making up for the lower accuracies in comparison to progeny testing and the dilemma on how to use genomically selected individuals in the four-way path scheme of cattle breeding.

The aim of this research was to test different scenarios of genomic selection in a realistic cattle population, such as Slovenian Brown Swiss, using a stochastic simulation. For this purpose, a holistic simulator of cattle populations under long-term selection was developed. The simulator comprises stochastic generation of the genome, animals and their phenotypes, evaluation of breeding values using different types of information, selection and mating.

MATERIAL AND METHODS

Simulation of a cattle population. A holistic simulator implementing all selection steps was developed. The simulator uses user-defined parameters to simulate a specific cattle population structure with overlapping generations. In this study initial 40 generations were simulated with AlphaSim software (Faux et al., 2016). This included the simulation of sequence data for 10 chromosomes from which 20,000 SNPs were used as causal loci affecting a polygenic trait (with heritability 0.25) and 20,000 SNPs were used as markers. In each of the next 20 generations we performed the following steps: a) estimated breeding values; b) selected the best individuals in line with the four-paths of selection; and c) mated selected individuals to obtain a new generation of individuals.

Estimation of breeding values. Blupf90 (Misztal et al., 2002) was used for the estimation of EBVs and gEBVs with the so called single-step method that utilizes phenotypic, pedigree, and genomic data jointly (Legarra, Aguilar, & Misztal, 2009). For the genomic scenarios, a reference population comprising ~11,000 cows and 100 progeny tested bulls was assumed. The female part of the reference population was updated every generation by replacing the oldest cows in the reference with randomly chosen cows from the current cow population.

Selection. Selection was performed with a Python script that implements selection following either conventional or genomic scenario. The script sets the size of the population, the selection intensity and generation interval for the four paths, further time parameters (the period of time individual animal persists in the population or AI), number of offspring per bull, the structure of the reference population for genomic selection, which individuals are genotyped, the evaluation criteria for choosing the bull for AI and for contracted mating, the strategy of the use of genomically tested (GT) bulls in the population and the number of selection cycles. All the stated parameters were set to mirror the Slovenian Brown Swiss population.

Scenarios. The simulated scenarios included a conventional and four genomic scenarios differing in the use of genomic information (Table 1). The scenarios differed in the male path of

selection only. Genomic information was used either as a criteria for the selection of bulls for progeny testing or for breeding in AI directly (PT* and GT in Table 1, respectively).

Table 1: Design of the breeding scenarios.

	Conventional	Genomic			
		A	B	C	D
Sires of sires	PT bulls	PT* bulls	PT bulls	GT bulls	GT bulls
Sires of dams	PT bulls	PT* bulls	GT bulls	PT bulls	GT bulls

PT = progeny tested, GT = genomically tested

*Young bulls selected based on genomic breeding values.

RESULTS AND DISCUSSION

Annual genetic gain expressed as the average breeding value in units of standard deviation (SD) and generation intervals for the five compared scenarios are shown in Figure 1. The conventional scenario had the lowest genetic gain (0.10 SD per year). This showed that although the accuracy of EBV in the conventional scenario is high (~0.75 for dams and ~0.99 for progeny tested males), it does not counterbalance the long generation intervals. The second lowest genetic gain was observed for the genomic scenario A (0.14 SD per year). This scenario was analogous to the conventional with the difference of using gEBV instead of PAs as criteria to enrol young bulls into progeny testing. Since the accuracy of gEBV is higher than that of PA a more accurate selection of the best male calves can be performed. Consequently, although the generation intervals in genomic A and conventional scenarios were equal, the increased accuracy of the early selection in genomic scenario increased genetic gain.

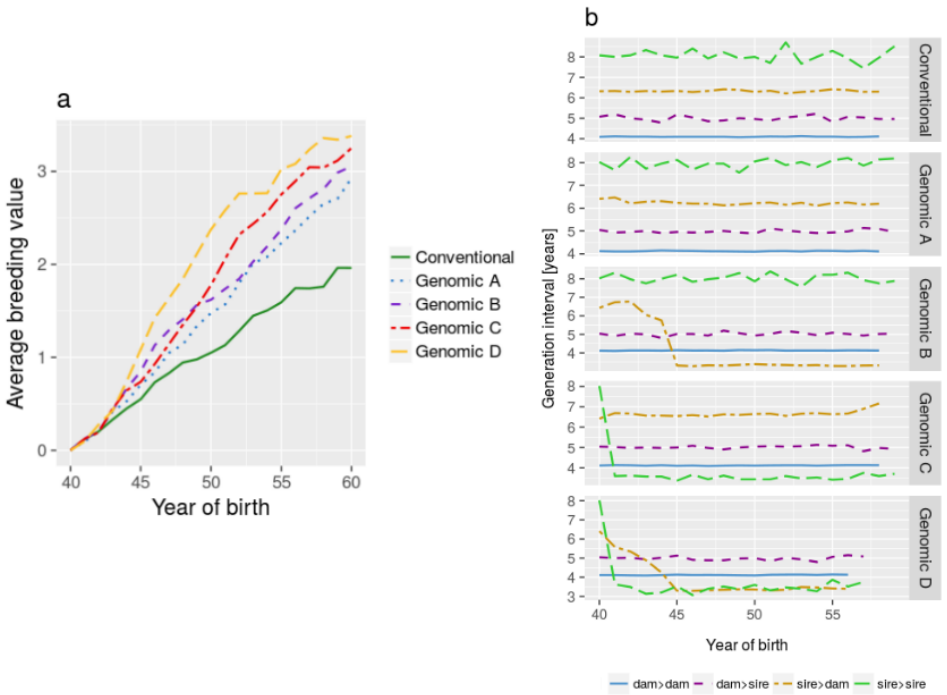

Figure 1: a) Genetic gain in units of genetic standard deviation b) Generation intervals for the four paths of selection for the tested scenarios.

Additional increase in genetic gain was achieved by reducing the generation interval (genomic scenarios B, C and D). When comparing genomic scenarios B and C with similar generation intervals the results showed a superiority of the genomic scenario C (0.15 and 0.17 SD per year, respectively for scenarios B and C). This could be explained with a larger decrease in generation interval (Figure 1b) when selecting sires of sires in scenario C (from 8.1 to 3.7 years) as compared to the selection of sires of dams in scenario B (from 6.3 to 4.1 years). The highest genetic gain was observed for the genomic scenario D (0.18 SD per year) in which the reduction in generation interval was the largest. This scenario used genomically tested males used on the entire female population (as sires of sires and as sires of dams). However, more replicates of simulated scenarios are required to estimate whether the differences between the genomic scenarios are statistically significant.

To conclude, this study showed that amongst the tested scenarios, the use of genomically tested males on the entire female population brings the highest genetic gain in a specific population mirroring Slovenian Brown Swiss population. Moreover, it showed that the main increase is achieved by using genomically tested males as sires of sires where the decrease of generation interval is the biggest. These results instruct breeders of the Slovenian Brown Swiss population on how to proceed with this new type of selection in the future.

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HORIZONTALY ACQUIRED PATHOGENICITY ISLAND PAI*usp* OF *Salmonella bongori* AND UROPATHOGENIC *Escherichia coli*, IS A NEW MEMBER OF THE TyrR REGULON

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POVZETEK

Zunajčrevesna uropatogena *Escherichia coli* (UPEC) je dobro poznana patogena bakterija, izolirana iz bolnikov z okužbami sečil, medtem ko *Salmonella bongori* le redko povzroča obolenja pri ljudeh in je navadno prisotna, kot komenzal hladnokrvnih živali. Bakterijski virulentni dejavniki so pogosto zapisani v otokih patogenosti (PAI). PAI so vrinjeni segmenti DNA, ki so lahko mobilni in vplivajo na sposobnost bakterije, da povzroči bolezen gostitelja in/ali da v gostitelju lažje preživi. Uropatogeni specifični protein (Usp) *E. coli* je genotoksin, ki deluje na sesalske celice in je prisoten pri UPEC sevih. Majhen otok patogenosti PAI*usp*, lociran v kromosomski intergenski regiji *aroP-pdhR* kodira *usp* in tri spodaj ležeče gene *imu1-3*, ki so pomembni za zaščito producenta pred lastnim toksinom. Ta študija je rezultirala v treh pomembnih zaključkih. Prvič, PAI*usp* je horizontalno pridobljen otok patogenosti, ki ni prisoten samo pri UPEC, ampak tudi v javno dostopnih genomih *S. bongori*. Drugič, predhodno ugotovljen divergentni promotor P3 gena *aroP* je izključno odgovoren za transkripcijo PAI*usp* iz UPEC in *S. bongori*, s čimer je *usp* postal najnovejši član regulona TyrR. Posledično je prisotnost aromatskih aminokislin v okolju pomembna pri patogenezi *S. bongori* in UPEC. Tretjič, regulatorna regija *aroP/usp* ima novo značilnost. Specifično, z ureo posredovano ekspresijo promotorja P3, ki je od proteina TyrR neodvisna, kar nakazuje na novo in zapleteno regulacijo *usp*. Ti rezultati so podlaga za globje razumevanje regulacije horizontalno pridobljenih genov in njihovo vključevanje v že obstoječe regulatorne mreže.

Ključne besede: UPEC, PAI, genotoksin, horizontalni genski prenos

ABSTRACT

Extraintestinal uropathogenic *Escherichia coli* (UPEC) is a well know pathogenic bacterium isolated from patients with urinary tract infections (UTIs), while *Salmonella bongori* is only rarely observed in human infections and is found as a commensal of cold-blooded animals. Bacterial virulence factor genes are frequently encoded in pathogenicity islands (PAIs), inserted DNA segments that can be mobile and affect host pathobiology as well as bacterial fitness. The *E. coli* uropathogenic specific protein (Usp) is a genotoxin active against mammalian cells and is associated with UPEC strains. A small pathogenicity island PAI*usp* located within the *aroP-pdhR* chromosomal intergenic region encodes *usp* and three associated downstream *imu1-3* genes required for protection of the producer against its own toxin. This study resulted in three significant conclusions. First, PAI*usp* is a horizontally acquired pathogenicity island that is present not only in UPEC but also in *S. bongori* publicly available genomes. Second, the previously identified divergent P3 promoter of *aroP* is solely responsible for PAI*usp* transcription from both UPEC and *S. bongori,* thus making *usp* the

newest member of TyrR regulon. Consequently, presence of aromatic amino acids in the environment is important in *S. bongori* and UPEC pathogenesis. Third, *aroP/usp* regulatory region exhibits a novel feature. In particular, TyrR independent urea induced expression of P3 promoter, which indicates on a novel and complex regulation of *usp*. These results provide a basis for a deeper understanding of horizontally acquired gene regulation and their integration into already existing regulatory networks.

Key words: UPEC, PAIs, genotoxin, horizontal gene transfer

SHORT LECTURES – POPULATION GENETICS

Mateja Janeš

POPULATION VARIABILITY OF TIBETAN TERRIER

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POPULATION VARIABILITY OF TIBETAN TERRIER

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IZVLEČEK

Tibetanski terier spada med pasme psov, ki izvirajo iz najstarejših populacij psov. Zaradi geografske osamitve in prepričanja, da bi prodaja teh psov povzročila nesrečo, je pasma ostala precej izolirana skozi stoletja. V začetku 20. stoletja prejšnjega stoletja je prvi par tibetanskih terierjev prišel v Evropo in prvo leglo izven Tibeta je bilo registrirano kot Lhasa terierji, kasneje pa je leta 1930 pasmo indijski kinološki klub preimenoval v tibetanskega terierja. Skozi zgodovino pasme sta nastali dve liniji tibetanskih terierjev, Lamleh in Luneville. Zahodna populacija tibetanskega terierja predstavlja relativno ozek genski sklad, ki so ga popestrile številne epizode križanj s psi podobnega izgleda. Imeli smo priložnost za zbiranje vzorcev v originalni populaciji tibetanskih terierjev v Tibetu in F1 križancev s predstavniki zahodne populacije tibetanskega terierija. V tej študiji smo analizirali 18 mikrosatelitnih lokusov in sekvencirali 5'-konec regulatorne regije mtDNA. Na podlagi naših rezultatov lahko potrdimo domnevo, zahodna populacija tibetanskih terierijev predstavlja samo majhen del genetske pestrosti v originalni populaciji v Tibetu.

Ključne besede: tibetanski terier, mikrosateliti, mtDNA, struktura populacije

ABSTRACT

The Tibetan terrier is considered to be one of the dog breeds descending from the most ancient dogs. Due to geographic isolation and wide spread belief that selling of these dogs would cause bad luck, the breed remained quite isolated over centuries. In the early 20iest of the last century, the first couple was brought to Europe and the first litter outside Tibet was registered as Lhasa Terriers, the name which has been changed by the Kennel Club of India to Tibetan terrier in 1930. During the history of the breed, two lines of Tibetan Terriers were established, Lamleh and Luneville. The western population represents a relative narrow gene pool, which has been enriched by several outbreeding episodes during the history of the breed with dogs of similar appearance. Recently, we got the opportunity to sample the original population of Tibetan terriers as well as F1 crosses with representatives of the western Tibetan terrier population. In the present study we analysed 18 microsatellite loci and sequenced the 5'-end of the mtDNA D-loop region. Based on our results, we can confirm the assumption that western Tibetan terrier population only represents a tiny part of genetic variation present in the indigenous population.

Key words: Tibetan terrier, microsatellites, mtDNA, population structure

INTRODUCTION

The first mention of the Tibetan Terrier in writing dates to 1895 when the breed was described as “neither more nor less than a rough terrier” and the Tibetan dog has thus been labelled a Tibetan Terrier. There are two linages of modern Tibetan Terrier breed in the Western World. In 1922, A.R.H. Greig, an English surgeon working as a medical missionary with the Women’s Medical Corps of India, near the border of Tibet acquired the first female and two years later also a male Tibetan Terrier. In 1930 she returned to England with this couple and established Lamleh Kennel. In 1937 she convinced the Kennel Club in England to recognize the Tibetan Terrier as its own exclusive breed. In 1953 John Downey found a stray dog on the docks of Liverpool, Great Britain Dusky. After being declared to be Tibetan Terrier by several British judges, Dusky was registered as a Tibetan Terrier by the English Kennel Club as Trojan Kynos in 1953. He and a the bitch Princess Aureus, daughter of another bitch Princess Chann who was found in Great Britain were the basis of the Luneville lineage. However, there is still the indigenous native population of Tibetan Terriers existing at the Tibetan plateau. Native Tibetan Terriers are believed to have evolved over the time from the North Kunlun Mountain Dog and the Inner Mongolian Dog. In that case, Tibetan Terrier would be the ancestor of most other dog breeds from Himalayas such as Lhasa Apso, Shih Tzu and Tibetan Spaniel. The Tibetan Terrier was bred and developed first in the monasteries of the Lost Valley of Tibet. Due to their geographic isolation and limited breeding and dispersal, Tibetan Terriers can be assigned to a very limited group of early foundation stock and genetic structure of native Tibetan Terriers should differ from genetic structure of their European lineages.

The genetic structure of the domestic dog has been investigated using mtDNA (Tsuda et al. 1997, Vilà et al. 1997, Vilà et al. 1999) or microsatellite markers (Koskinen and Bredbacka 2000, Oberbauer et al. 2003, Parker et al. 2004). The variation observed in mtDNA indicates that all dogs originate from a common gene pool. Parker deconstructed the relationships among 414 dogs representing 85 breeds using a Bayesian model-based clustering algorithm (Pritchard, Stephens and Donnelly 2000) to identify genetically distinct subpopulations based on microsatellite allelic frequency patterns. This algorithm assigns most dogs to unique breed-specific clusters.

The aim of this study was to elucidate the genetic variability in the native Tibetan Terriers and their western lineages using mtDNA and autosomal microsatellite markers.

MATERIAL AND METHODS

Sample collection and DNA extraction. We collected 68 buccal swabs from Tibetan Terriers representing the native population of Tibetan Terrier (25), two European lineages: lameh (21) and luneville (4) and three different filial generations of native x lamleh mixes: F1 generation (8), F2 generation (6) and F3 generation (4). Samples were collected in Croatia, Slovenia, and in Tibet. DNA was isolated using DNeasy Blood & Tissue Reagent Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions.

Microsatellite analysis. We examined 18 microsatellite markers suggested by the International Society of Animal Genetics (ISAG): AHTK211, CXX279, REN169O18, INU055, REN54P11, INRA21, AHT121, FH2054, REN162C04, AHT137, REN169D01, AHTH260, AHTK253, INU005, AHTH171 and REN247M23, using the reagent kit (Canine Genotyping Panel 1.1, Thermo Scientific, California, USA). Genotyping was performed on an automated capillary sequencer ABI 3130xl. The microsatellite alleles were called using GENEMAPPER v 4.0 (ABI, Foster City, CA, USA) and manually checked.

We used the R software, version 3.2.3 (Team 2017) to calculate the number of different alleles per lineage, mean Shannon’s information index, observed heterozygosity, expected

heterozygosity, inbreeding coefficient, inbreeding coefficient of an individual relative to the total population and genetic divergence among subpopulations. The principal component analysis (PCA) was carried out on the microsatellite genotypes to determine breed lineage relationships. PCA was performed using pca3d and plyr (Wickham 2011) packages for R. To determine the true number of genetic populations represented by the sampled dogs and to assign individuals to identified clusters, we used STRUCTURE (Pritchard et al. 2000). A model with assumption of admixture and correlated allele frequencies was used for all STRUCTURE runs, with a burn-in of 10,000 iterations followed by 100,000 MCMC iterations. Runs were repeated six times for each K value of K=1-10.

Mitochondrial DNA analysis. The 680 base pair mtDNA control region was amplified using primers and PCR conditions previously described by Boyko et al. (2009). The mtDNA sequences were aligned using MUSCLE algorithm in MEGA 7.0.26 (Kumar, Stecher and Tamura 2016). Dendrograms based on genetic distances between breeds were obtained by the Neighbor-Joining Tree clustering (Sneath and Sokal 1973) implemented in MEGA 7.0.26. A network analysis was used to visualize the spatial distribution of the sequence variation among the different mtDNA haplotypes. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei and Kumar 2004). The analysis involved 49 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 602 positions in the final dataset. Evolutionary analyses were conducted on native population of Tibetan Terriers branched together on two distant branches with two samples of F1 filial generation of mix between native and Lamleh lineage of Tibetan Terriers.

RESULTS AND DISCUSSION

Genetic diversity. For the 68 Tibetan Terriers 18 autosomal microsatellites, Na mean I, (Ho), (He), (Fis), (Fit) and (Fst) were counted (Tab. 1). The native lineage showed the highest Na (117) while luneville showed the lowest (90). Native population of Tibetan Terrier had Na between 6 and 8, lamleh between 4 and 8 and luneville between 4 and 7. Locus FH2054 had the highest Na in native and lamleh but locus REN169O18 in luneville. The highest mean I was counted for locus AHT137 and the lowest for INU030. The highest Ho was calculated for locus AHTk211 while the highest He was for locus FH2054. The highest Fis was counted for locus AHTk253, Fit for loci INU005 and REN54P11 and Fst for loci REN54P11 and INU030.

Table 1: Microsatellite analysis: number of different alleles per lineage (Na), mean Shannon's information index (Mean I), observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficient (Fis), inbreeding coefficient of an individual relative to the total population (Fit) and genetic divergence among subpopulations (Fst)for 18 microsatellite markers.

Locus	Na			Mean I	Ho	He	Fis	Fit	Fst
	native	lamleh	luneville						
AHTk211	7	6	5	1,05	1	0.76	–0,15	–0,08	0,07
CXX279	6	7	5	1,34	0.81	0.7	–0,16	–0,06	0,08
REN169O18	7	7	7	1,51	0.69	0.74	0,05	0,12	0,07
INU055	6	5	5	1,12	0.65	0.74	–0,05	0,01	0,06
REN54P11	6	5	4	1,22	0.75	0.7	0,01	0,15	0,15
INRA21	6	6	4	1,34	0.88	0.77	–0,15	–0,04	0,10
AHT137	6	6	5	1,77	0.88	0.73	–0,08	0,01	0,08
REN169D01	6	5	5	1,18	0.66	0.72	–0,16	–0,03	0,11
AHTh260	7	7	6	1,48	0.78	0.79	–0,12	–0,02	0,09
AHTk253	6	6	5	1,21	0.53	0.69	0,06	0,13	0,07
INU005	7	6	5	1,37	0.63	0.74	0,04	0,15	0,11
INU030	7	5	5	0,99	0.63	0.76	–0,14	0,03	0,15
FH2848	6	5	5	1,29	0.71	0.73	–0,11	–0,04	0,06
AHT121	7	6	5	1,66	0.94	0.78	–0,14	–0,04	0,09
FH2054	8	8	5	1,41	0.71	0.8	–0,02	0,08	0,10
REN162C04	7	7	5	1,52	0.71	0.74	–0,02	0,08	0,10
AHTh171	6	6	5	1,45	0.85	0.74	–0,09	–0,01	0,07
REN247M23	6	4	4	1,22	0.76	0.74	–0,14	0,01	0,12
	117	107	90						

PCA analysis. The results of PCA reveal differentiation between lineages (Fig.1). European lineages (Lamleh and Luneville) are similar to each other but native lineage is clearly different. F1, F2 and F3 populations are placed between the native and European lineages as expected, where F1 is in the middle between Lamleh and native, but F2 and F3 are closer to Lamleh because of higher proportion of Lamlech in later filial generations backcrossed with lamleh.

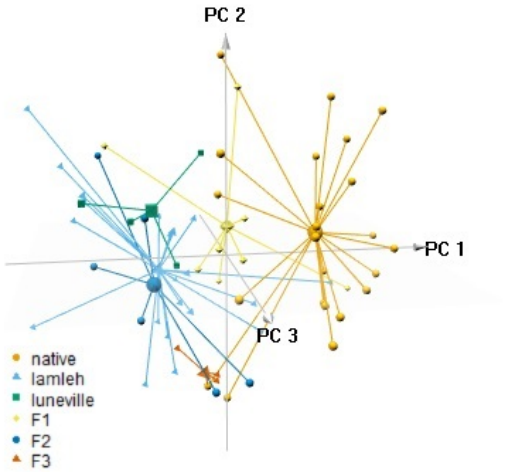

Figure 1: PCA analysis performed using pca3d package in R. Legend shows the names of different lineages of Tibetan Terriers.

Genetic distances and clustering. The evolutionary history was inferred using the Neighbor-Joining algorithm. The optimal tree with the sum of branch length = 0.05854153 is shown (Fig.2). European lineages Lamleh and Luneville branched together with other samples of F1, F2 and F3 filial generations of native x Lamleh cross.

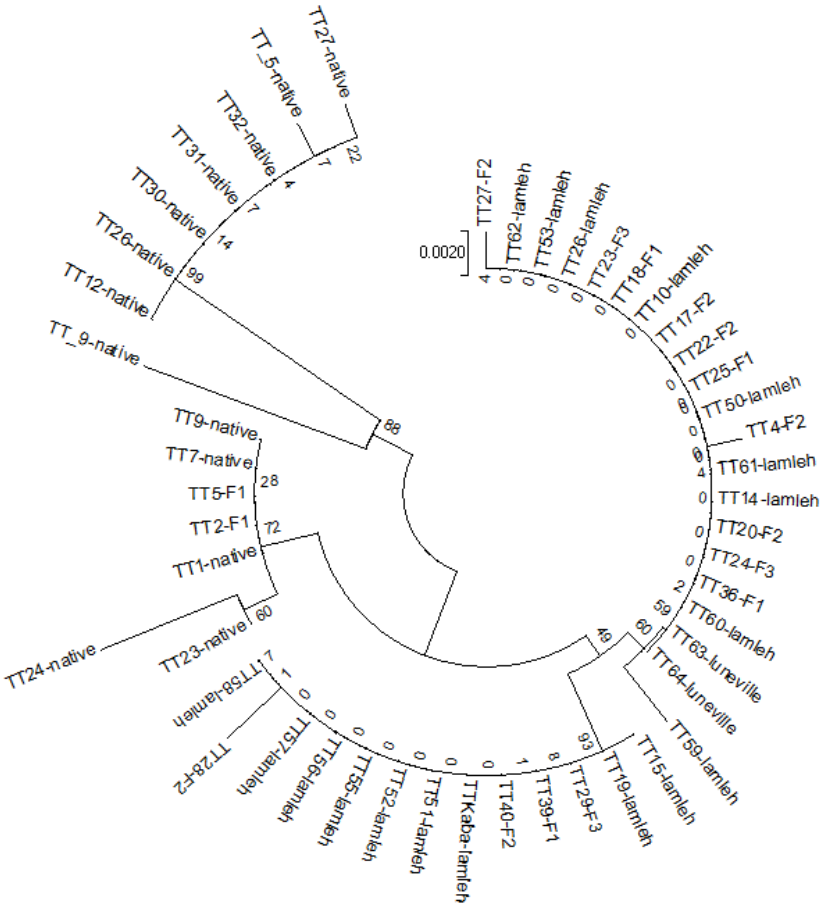

Figure 2: Evolutionary relationships of linneages using the Neighbor-joining method.

STRUCTURE analysis. The results of STRUCTURE analysis for K=2 is shown in Figure 3. This analysis showed that within a population of Tibetan Terrier the dogs were genetically very similar. The native Tibetan Terriers showed almost 5% contribution of one subpopulation and 95% contribution of the second subpopulation. European lamleh and luneville lineages showed the opposite results, 95% contribution of the second and 5% contribution of the first subpopulation, whereas, F1 cross showed between 20% and 80% of each subpopulation, F2 showed 90% of the second subpopulation, and F3 almost 100% of the second subpopulation.

STRUCTURE HARVESTER software was used to determine the most likely value of K to 2. Therefore, although different, our 6 populations of Tibetan Terriers actually consist of two clusters – native Tibetan, and European cluster. Genetic basis of the native population from Tibetan plateau is wider than population of European lineages Genetic variability of native population of Tibetan Terrier is not proportionally reflected in the phenotype. The existence of the broader genetic pool in the native population is important for possible prevention of the increase in homozygosity and potentially of genetic diseases.

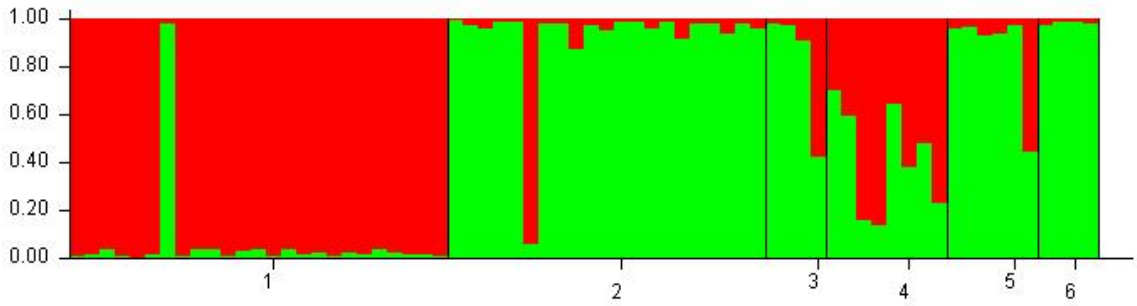

Figure 3: STRUCTURE analysis of 6 population groups of Tibetan Terrier breed from Europe and Tibet. The results are shown for a typical run with K=2. Each vertical column represents a different dog, whereas green and red color represent two different subpopulations in the admixture. The x axis numbers are 6 population groups: 1 – native Tibetan Terriers, 2 – European lamleh lineage, 3 – European luneville lineage, 4 – F1 mix generation, 5 – F2 mix generation, 6 – F3 mix generation. The y axis shows the proportional of the genotype attributed to each subpopulation for each dog.

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SHORT LECTURES – INTERACTIONS GENOME-ENVIRONMENT

Luka Predojević

COMPARATIVE ANALYSIS OF PHYLOGENETIC ASSIGNMENT OF HUMAN AND AVIAN EXPEC AND FECAL COMMENSAL STRAINS OF *Escherichia coli* USING BOTH CLERMONT PCR PHYLOTYPING METHODS REVEALED A NEED FOR A NEW PCR PHYLOTYPING MEHTOD

Katja Molan

EXTENDED-SPECTRUM β-LACTAMASE PRODUCING *Escherichia coli* AND EMERGENCE OF CLONAL GROUP ST131 AMONG HUMAN RESPIRATORY TRACT ISOLATES

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COMPARATIVE ANALYSIS OF PHYLOGENETIC ASSIGNMENT OF HUMAN AND AVIAN EXPEC AND FECAL COMMENSAL STRAINS OF *ESCHERICHIA COLI* USING BOTH CLERMONT PCR PHYLOTYPING METHODS REVEALED A NEED FOR A NEW PCR PHYLOTYPING MEHTOD

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POVZETEK
Bakterija Escherichia coli (*E. coli*) je sicer pretežno komenzalni mikroorganizem in del normalne črevesne mikrobiote številnih živali in človeka, a je lahko tudi patogena in zmožna povzročitve črevesnih in zunajčrevesnih infekcij. Raziskave so pokazale, da je filogenetska skupina *E. coli* povezana s tipom *E. coli*. Za filogenetsko klasifikacijo tipov *E. coli* sta poznani dve metodi, temelječi na PCR, in sicer metoda tripleks PCR in metoda razširjen kvadrupleks PCR, obe prvič opisani s strani Clermont in sod. (2000, 2013). Prva metoda, tripleks PCR, na podlagi ustreznih označevalcev v genomu, omogoča razporeditev sevov *E. coli* v štiri filogenetske skupine in sicer A, B1, B2 in D, med tem ko, novejša, prenovljena metoda, razširjen kvadrupleks PCR, ta nabor razširja še na štiri nove filogenetske skupine in sicer C, E, F in klad I/II. Primerjalna analiza razporeditve v filogenetske skupine različnih sevov *E. coli*, človeških in ptičjih zunajčrevesnih sevov ter fekalnih komenzalnih sevov z obema metodama, je pokazala prehod nekaterih sevov iz ene v drugo filogenetsko skupino, a tudi v skupino sevov, ki se jih z razširjeno kvadrupleks metodo ne da uvrstiti v nobeno od filogenetskih skupin. V naši raziskavi smo zasledili še večji odstotek takih neuvrščenih sevov. Pregled lliterature je razkril, da so tudi nekateri drugi raziskovalci dobili visoke odstotke neuvrščenih sevov. Dobljeni rezultati tako narekujejo iskanje in vpeljavo novih metod, ki bi na podlagi novih označevalcev uspešno uvrščale seve *E. coli* v filogenetske skupine.

Ključne besede: *E. coli*, filogenija, PCR, klasifikacija

ABSTRACT
Escherichia coli (*E. coli*) is mostly a commensal microorganism and part of normal gut microbiota of numerous animals and humans, however it can be also pathogenic and capable of causing intestinal and extraintestinal infections. Studies showed that the *E. coli* phylogenetic group is linked with the type of *E. coli*. For phylogenetic classification of *E. coli* two PCR based methods are known – the triplex and the extended quadruplex PCR, both described by Clermont et al. (2000, 2013). The first method enables assignment of *E. coli* strains to four different phylogenetic groups: A, B1, B2 and D, while the second, revised, newer method expands the spectrum to four additional groups: C, E, F and clade I/II. Comparative analysis of phylogroup assignment of human and avian extraintestinal *E. coli* strains as well as fecal commensal *E. coli* using both mentioned methods showed reclasifications of strains into other phylogenetic groups and even a small percentage of non-typeable strains (Logue et al., 2017). In our study an even higher percentage of non-typeable strains was found. Literature search revealed that also some other researchers had high percentages of non-typeable strains.

Thus, the obtained results exposed the need for a new method that would, based on new markers, enable efficient classification of *E. coli* strains into phylogenetic groups.

Key words: *E. coli*, phylogeny, PCR, classification

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EXTENDED-SPECTRUM β-LACTAMASE PRODUCING *Escherichia coli* AND EMERGENCE OF CLONAL GROUP ST131 AMONG HUMAN RESPIRATORY TRACT ISOLATES

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POVZETEK

Escherichia coli (*E. coli*) je zelo raznolika, predvsem zaradi različnega nabora genov, povezanih z virulenco. Posledično je vpletena v različne črevesne in zunajčrevesne bolezni. Zunajčrevesne patogene *E. coli* (ExPEC) pri ljudeh pogosto povzročajo vnetje urinarne poti in tudi bakteriemije. Pri pticah povzročajo kolibaciloze, ki se po navadi začnejo v dihalih. Okužbe dihal z *E. coli* so pri ljudeh redke, a se delež v zadnjih letih povečuje. Okužbe z ExPEC so postale globalen problem predvsem zaradi širitve virulentnih in rezistentnih klonov, kot je ST131. *E. coli*, ki proizvaja β-laktamaze z razširjenim spektrom delovanjem (ESBL), je bila predmet več raziskav, v katerih so se osredotočili predvsem na izolate iz okužb urinarne poti in bakteriemije. Namen naše raziskave je bil ugotoviti ali izolati ESBL-*E. coli* iz dihal, glede na filogenetsko skupino in nabor genov povezanih z virulenco, spadajo v posebno skupino ExPEC. Analizirali smo seve izolirane iz bolnikov slovenske največje bolnišnice v obdobju 12 let (2002–2013). Izolate smo uvrstili v filogenetske skupine in jih preverili za prisotnost genskih zapisov za dejavnike virulence ter zapisov za odpornost proti antibiotikom (izbrani zapisi za ESBL in plazmidno posredovani zapisi za odpornost proti kinolonom-PMQR). Zanimalo nas je, kdaj in če sploh, se je med izolati pojavil ST131 in v kakšnem odstotku. V zbirki izolatov ESBL se je klon ST131 začel pojavljati po letu 2005. Izolati ST131 so izkazali robusten profil virulentnih dejavnikov in so se v večini uvrstili v filogenetsko skupino B2.

Ključne besede: *E. coli*, ESBL, ST131, človeška dihalna pot

ABSTRACT

E. coli exhibits considerable genetic diversity due to a wide range of virulence associated genes and is consequently implicated in a variety of intestinal and extraintestinal diseases. ExPEC is the major cause of urinary tract infections and a common cause of bacteremia. In birds *E. coli* strains frequently cause colibacillosis, a disease that usually starts in the respiratory tract. However, human respiratory tract infections due to *E. coli* are uncommon, although increasing in recent years. ExPEC infections have become, due to the emergence of highly virulent and antimicrobial-resistant clones as ST131, a serious world health problem. Several studies focused on the genetic characteristics of ESBL-*E. coli*, predominantly including isolates from urinary tract infections and bacteremia. The aim of our study was to reveal whether ESBL-*E. coli* isolated from respiratory tract infections are a special group of ExPEC strains with respect to phylogenetic groups and virulence associated genes. Further, we were interested in emergence of ST131 isolates. To fulfill the aim, we determined and analyzed the phylogenetic background, ESBL- and PMQR- group, virulence profile and prevalence of ST131

among ESBL-*E. coli* isolates from respiratory tract specimens gathered over a period of 12 years (2002–2013) in the largest Slovenian hospital. Our findings provided evidence for emergence and high prevalence of clone ST131 among respiratory tract isolates isolated after 2005. ST131 isolates were predominantly from phylogenetic group B2 and exhibited a robust virulence gene profile.

Key words: *E. coli*, ESBL, ST131, human respiratory tract

CLOSING LECTURE

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GENE THERAPY OF CANCER

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POVZETEK

Elektroporacija je metoda dostave zdravil in genskega materiala v celice *in vitro* ali tkiva *in vivo,* ki poveča prepustnost membrane in tako omogoči vstop molekulam, ki sicer ne morejo vstopiti v celico (npr. siRNA, pDNA in nekateri citostatiki). Trenutno se elektrokemoterapija (kombinacija elektroporacije in citostatika) uporablja za zdravljenje različnih vrst tumorjev. Z namenom, da bi povečali lokalno protitumorsko učinkovitost elektrokemoterapije, lahko uporabimo genski elektroprenos z imunomodulatornim učinkom, kot adjuvantno terapijo.

Interlevkin 12 (IL-12) je citokin, ki ima protitumorske in antiangiogene učinke, posredovane z indukcijo prirojenega in pridobljenega imunskega odziva. Genski elektroprenos IL-12 ima tako lokalne, kot tudi sistemske učinke in sicer na oddaljene tumorje, ki niso bili zdravljeni. Študije so pokazale do 100 % odziv tumorjev na terapijo. Tak pristop zdravljenja se je v ameriški klinični študiji izkazal kot učinkovit za zdravljenje kožnih metastaz melanoma.

Ker elektrokemoterapija lahko povzroči imunološko celično smrt, bi se lahko kot terapevtski pristop uporabil adjuvantni genski elektroprenos v peritumoralno tkivo (imunogeni elektroprenos), ki bi imel loko-regionalni učinek in učinek na oddaljene metastaze. Zato predlagamo kombinacijo elektrokemoterapije s peritumoralnim genskim elektroprenosom IL-12, da bi potrdili domnevo, da lahko za uspešno zdravljenje tumorjev uporabimo elektrokemoterapijo, ki bi bila okrepljena z imunogenskim elektroprenosom, kot *in situ* vakcinacijo.

Ključne besede: genska terapija, elektroporacija raka, elektrokemoterapija, genski elektroprenos, interlevkin 12

ABSTRACT

Electroporation is a platform technology for drug and gene delivery. When applied to cell *in vitro* or tissues *in vivo*, they lead to increase in membrane permeability for molecules which otherwise cannot enter the cell (e.g. siRNA, pDNA and some chemotherapeutic drugs). Based on the current knowledge, electrochemotherapy (a combination of drug and electric field pulses) is used for tumor treatment and has shown great potential. With an attempt to increase local tumor effectiveness of electrochemotherapy, electrotransfer of genes with immunomodulatory effect can be used as adjuvant treatment.

Interleukin 12 (IL-12) is a cytokine that has antitumor and antiangiogenic effects, which are mediated through the induction of innate and adaptive immune response. Intratumoral IL-12 gene electrotransfer has local and also systemic effect on distantly growing tumors that were not treated. The studies demonstrated up to 100% tumor curability. This treatment approach has already been

tested in the clinical study in the USA, and has proved to be effective in treatment of skin melanoma metastases.

Furthermore, since electrochemotherapy can induce immunologic cell death, adjuvant gene electrotransfer to peritumoral tissue (immunogene electrotransfer) could be used as a therapeutic approach that would have locoregional effect as well as the effect on distant metastases. Therefore, we propose combination of electrochemotherapy with peritumoral IL-12 electrotransfer, as a proof of principle, showing that electrochemotherapy boosted with immunogene electrotransfer can be used as *in situ* vaccination for successful tumor treatment.

Key words: gene therapy, cancer electroporation, electrochemotherapy, gene electrotransfer, interleukin 12

POSTERS

POSTERS – BIOTECHNOLOGY

Anja Domadenik
DEVELOPMENT OF A GENETIC TEST FOR THE FELINE AB BLOOD GROUP SYSTEM

Miha Slapnik
SEX DETERMINATION IN HEMP (*Cannabis sativa L.*) USING DNA MARKERS

Sinja Svetik
ATTEMPT TO INDUCE HAPLOIDS OF CANNABIS (*Cannabis sativa* L.) WITH *in vivo* GYNOGENESIS TECHNIUQE USING IRRADIATED POLLEN

Špela Mestinšek Mubi
TISSUE CULTURE OF HEMP (*Cannabis sativa* L.): MICROPROPAGATION AND ADVENTITIOUS REGENERATION

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DEVELOPMENT OF A GENETIC TEST FOR THE FELINE AB BLOOD GROUP SYSTEM

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IZVLEČEK

Sistem krvnih skupin pri mačkah temelji na prisotnosti N-glikonevraminske (Neu5Gc) ali N-acetilnevraminske kisline (Neu5Ac) na površini rdečih krvnih celic. Encim citidin monofosfo-N-acetilnevraminska kislinska hidrolaza (*CMAH*) pretvarja Neu5Ac v Neu5Gc. V odvisnosti od funkcionalnosti tega encima, ločimo tri krvne skupine pri mačkah: A, B in AB. Določanje krvnih tipov mačk je klinično pomembno zaradi naravno prisotnih aloprotiteles, ki lahko povzročijo resne zaplete pri transfuziji ali neonatalno izoeritrolizo. Na voljo je več seroloških kompletov za določanje krvnih skupin pri mačkah, vendar se še vedno pojavljajo težave s tipizacijo. Ker te lahko privedejo do akutnih hemolitičnih reakcij, je priporočljivo genetsko testiranje (tudi za odkrivanje genotipa pri znanem serotipu). Insercija dolžine 18 bp, ki se nahaja 53 bp od eksona 1 gena *CMAH*, je bila identificirana kot verjeten vzrok za različne krvne skupine. Insercija povzroči premik odprtega bralnega okvirja (angl. open reading frame; *ORF),* ki vodi k zmanjšanju funkcionalnosti encima. V tej študiji smo razvili test na osnovi metode PCR-RFLP in določili genotip gena *CMAH* pri 28 mačkah različnih pasem. Pri 17 mačkah (60,7 %) insercije ni bilo, pri osmih mačkah (28,6 %) je bila insercija prisotna pri enem alelu, pri treh mačkah (10,7 %) pa pri obeh alelih. Genetsko testiranje omogoča odkrivanje heterozigotov, ki jih z uporabo aglutinacijskih testov ne zaznamo, zato je pomembno za preprečevanje neonatalne izoeritrolize, transfuzijskih reakcij in za selekcijo pri vzreji mačk.

Ključne besede: *CMAH*, hemolitična reakcija, krvne skupine pri mačkah, vzreja mačk

ABSTRACT

The feline blood type system depends on the presence of N-glycolylneuraminic (Neu5Gc) or N-acetylneuraminic acid antigen (Neu5Ac) on the surface of red blood cells. An enzyme, cytidine monophospho-N-acetylneuraminic acid hydrolase *(CMAH*) transforms Neu5Ac to Neu5Gc. Depending on this enzyme's functionality three feline blood types can be differentiated: A, B and AB. The feline blood typing is clinically important because of naturally occurring alloantibodies, causing potentially life-threatening complications in case of blood transfusion or neonatal isoerythrolysis. Several serological blood typing kits are available, however blood typing difficulties still occur. Since they can lead to acute haemolytic reactions, genetic testing is proposed (also to reveal the genotype behind the serotype). The insertion of 18 bp, located 53 bp upstream of exon 1 of *CMAH* gene, has been reported as a presumable cause of different feline blood types. It results in an open reading frame (ORF) shift leading to the lack of enzyme's functionality. In the present study, we developed a PCR-

RFLP method-based test and genotyped the insertion within *CMAH* gene in 28 cats of different breeds. We identified 17 cats (60.7%) without the insertion, 8 cats (28.6%) with the insertion in one allele and 3 cats (10.7%) with the insertion in both alleles. Genetic testing enables detection of heterozygotes, which are undetectable using agglutination tests, and can be used for prevention of neonatal isoerythrolysis, transfusion reactions and for selective breeding.

Key words: cat breeding, *CMAH,* feline blood types, haemolytic reaction

INTRODUCTION

The major blood group system in domestic cat has been discovered early in the 20th century (Ingebrigtsen 1912, Ottenberg and Thalhimer 1915). Feline AB blood group system is primarily defined by two alleles, A and B, which are associated with the presence of particular antigens on the surface of red blood cells in cats (Auer, Bell, and Coates 1982). These antigens are N-glycolylneuraminic (Neu5Gc) or N-acetylneuraminic acid antigen (Neu5Ac). An enzyme, cytidine monophospho-N-acetylneuraminic acid hydrolase (*CMAH*), plays a crucial role in transformation of Neu5Ac to Neu5Gc (Muchmore et al. 1989). Depending on the enzyme's functionality there are three blood types: A, B and AB. Blood type A is a result of a fully functioning *CMAH*, which results in predominance of Neu5Gc antigen and a small amount of Neu5Ac antigen. In the case of blood type B, *CMAH* is not functional, hence only Neu5Ac antigen can be detected. In the case of the AB blood type the enzyme can still carry out enzymatic reaction, but at lower level of functionality, therefore some of antigens are Neu5Ac and some are Neu5Gc.

However, *CMAH* gene is non-functional in humans (Hayakawa et al. 2006, Chou et al. 1998). This loss-of-function mutation is explained by an evolutionary defence mechanism to fight pathogens (Ségurel et al. 2012, Varki 2001), but negative selection towards allele B in cats was also explained by the occurrence of neonatal isoerythrolysis (Giger, Bucheler, and Patterson 1991). This is a result of maternal immune response to foreign antibodies during gestation (Cain and Suzuki 1985, Giger and Casal 1997, Hubler et al. 1987). Therefore, the life of an A or AB blood type kitten born to a B-type queen can be at risk due to the fact that it receives anti-A alloantibodies through the colostrum for the first 16 hours of its life (Giger and Casal 1997).

In feline blood system, there are some additional differences compared to blood system in humans or other species. All cats with B blood type develop a high titre of anti-A alloantibodies at a few weeks of age, while only few of A blood type cats develop quantifiable titres of anti-B alloantibodies. This is the reason for the possible occurrence of life-threatening haemolytic transfusion reactions, when it comes to transfusions of AB-mismatched blood (Auer, Bell, and Coates 1982, Giger 1992, Giger and Casal 1997, Euler et al. 2016). A variety of agglutination based serological blood tests are accessible, that determine a blood type by naturally occurring alloantibodies (Holmes 1950, Andrews et al. 1992).

However, besides the counted blood typing difficulties, those tests are not able to detect heterozygotes. Genetic testing is therefore a reasonable proposal. DNA variants of *CMAH* gene have been reported; 7 silent and 4 missense mutations in exons 2 (G139A or G142A, T265A), 4 (A324C) and 13 (G1600A). Besides that, we can also find additional 6 mutations upstream of exon 1 (G-108A, G-217A, C-371T, A-498G, G-539A and 18bp ins -53) (Bighignoli et al. 2007, Gandolfi et al. 2016).

The aim of the present study was to develop a genetic test for feline blood typing based on the 18 bp insertion on site 53 upstream of *CMAH* exon 1, which results in an ORF shift leading into

lack of enzyme's functionality. It is useful as a diagnostic test in case of neonatal isoerythrolysis, for cases of transfusion and for breeding purposes.

MATERIAL AND METHODS

Animals and DNA extraction. Blood samples of 28 cats of various breeds were obtained; Exotic Shorthair (3), Scottish Fold (3), Persian (4), British Shorthair (1), Maine Coon (7) and Ragdoll (10). The DNA extraction was performed using Isolate II Genomic DNA Kit (Bioline) following the manufacturer's instructions.

PCR-RFLP analysis. Polymerase chain reaction (PCR) was performed to screen for 18 bp insertion in the *CMAH* gene, using the following primer pair: forward: 5'-CTGAAGCAACACAGCAGAGG -3' and reverse: 5'- AGTGTTGGTCTCGGGTTCCT -3'. Primers were designed using Primer3Plus tool (Untergasser et al. 2007). Depending on the genotype, there were 2 possible lengths of PCR products; 195 bp for allele A (without the insertion) and 213 bp for allele B (18 bp insertion). The amplification reactions were performed as follows: 5 min at 95 °C, followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 15s, and final elongation step at 72 °C for 2 min. The reaction volume was 20 µl and contained 5 x MyTaq reaction buffer (Bioline) containing dNTPs (5 mM) and $MgCl_2$ (15 mM), 0.35 µM primers, 0.0625 U/µl MyTaq DNA polymerase (Bioline), and 1µl template DNA. PCR product was digested using restriction endonuclease *Mwo*l (R0573, New England Biolabs) to obtain fragments of 112 bp and 101 bp in case of the insertion (213 bp product) or uncut fragment in case of 195 bp allele. The restriction reaction consisted of 13 µl PCR product, 1.8 µl restriction buffer, 2.7 µl H_2O, and 0.5 µl (5U/µl) of the enzyme, incubated overnight at 37 °C. Fragments after restriction were analysed on 3 % agarose gel, stained with ethidium bromide. The design of the genetic test is presented in Figure 1.

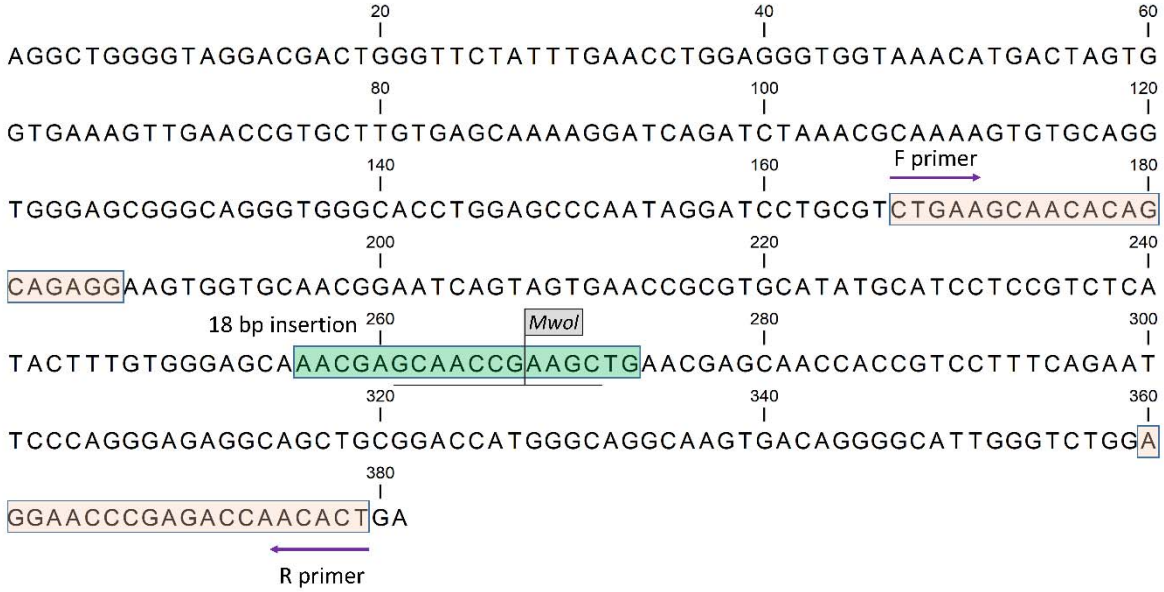

Figure 2: The visual presentation of primer annealing sites, 18 bp insertion and Mwol restriction site in a segment of CMAH exon 1 and the flanking intronic region.

RESULTS AND DISCUSSION

Genotypes. With PCR-RFLP analysis, we identified 17 cats (60.7%) without the insertion, 8 cats (28.6%) with the insertion in one allele and 3 cats (10.7%) with the insertion in both alleles. The

observed distribution of predicted phenotype frequency in cats is consistent with previous studies from other feline breeds (Giger, Bucheler, and Patterson 1991). Detailed results are presented in Table 1.

Table 2: Blood types of analysed cats. Insertional mutation: wild type (+/+), heterozygous (+/–), homozygous (–/–). Genotype: The "A" and "Ab" alleles are dominant over "b" allele. Cats with genotypes AA and Ab will be type A, homozygous bb will be type B. The type AB, occurs rarely, its heritability is not understood.

FELINE BREED	*INSERTIONAL MUTATION*	*GENOTYPE*	*PREDICTED BLOOD TYPE*	*NUMBER OF OBSERVED ANIMALS*
Maine coon	+/+	AA	A	5
	+/–	Ab	A	2
Ragdoll	+/+	AA	A	9
	–/–	Bb	B	1
Persian	+/+	AA	A	3
	–/–	Bb	B	1
Exotic Shorthair	+/–	Ab	A	2
	–/–	Bb	B	1
Scottish Fold	+/–	Ab	A	3
British Shorthair	+/–	Ab	A	1
Σ				28

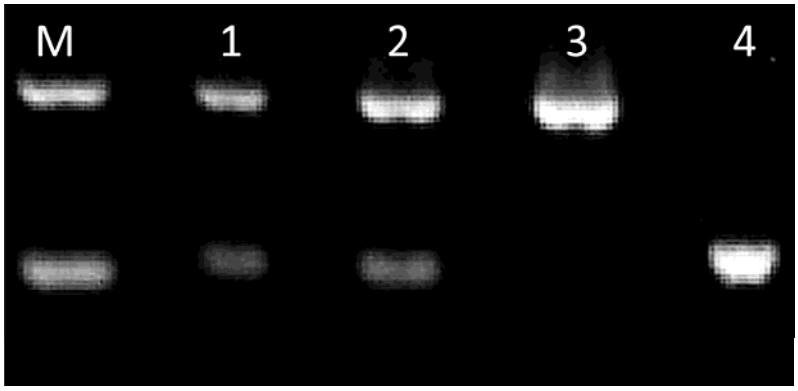

Figure 3: Example of PCR-RFLP fragments on agarose gel for the three different genotypes of CMAH locus (M: standard 100 bp marker; 1,2: heterozygote; 3: homozygote for A allele; 4: homozygote for B allele)

Feline AB blood group is a very complex blood system that has not yet been fully clarified. In the present study, we confirmed the involvement of the 18 bp *CMAH* gene insertion in the determination of feline blood types, which has already been proposed in the literature (Bighignoli et al. 2007). This insertion causes the production of a non-functional enzyme, which leads to its incapability to transform Neu5Ac to Neu5Gc. Non-functional enzyme is generated due to formation of stop codon at position 54 which leads to termination of the translation (Bighignoli et al. 2007). As mentioned before, Neu5Gc antigen is associated with A blood type and Neu5Ac antigen is associated with B

blood type. Most cats have A or B blood types and according to that we can explain the heredity of feline AB blood type system as dominant for allele A (Bighignoli et al. 2007). In the case of bb genotype, we can assure that a cat has B blood group, since none of its alleles can produce a functional enzyme. Anyway, there is an additional, mostly rare blood type, that has been observed in cats and it is called AB blood type (Gandolfi et al. 2016, Bighignoli et al. 2007). The latter is a result of the presence of A allele and a codominant B allele. Its mode of inheritance is currently unclear, due to apparently different inheritance patterns in different breeds (Griot-Wenk et al. 1996). Therefore, numerous proposals of wide variety of SNPs and other alterations that generate different *CMAH* variants, have been suggested as the cause of AB phenotype (Gandolfi et al. 2016, Bighignoli et al. 2007, Omi et al. 2016). As stated by that, the 18 bp insertion, analysed in this study, could also cause AB blood type phenotype. This could be discovered through already established agglutination tests (Andrews et al. 1992). It is essential to notice that genetic testing of feline blood types enables the differentiation between AA and Ab genotypes, which is a limiting factor in agglutination assays that are solely phenotypic. A future perspective would therefore be to combine agglutination test methods with genetic analysis of feline samples. This would be of good assistance for further clarification of feline blood types. Distribution of different blood types of samples tested in this study is wide, which is consistent with prior research (Gandolfi et al. 2016, Bighignoli et al. 2007). Diversity in blood types is especially important for conservation of rare breeds of cats which can be controlled by careful selection. In order to perform artificial selection, establishment of reliable genetic test is required. Here we provide a step forward in this area, which can also contribute to resolve complications during transfusion and gestation.

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SEX DETERMINATION IN HEMP (*Cannabis sativa* L.) USING DNA MARKERS

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POVZETEK

Ključna lastnost, povezana s pridelavo in žlahtnjenjem navadne konoplje (*Cannabis sativa* L.), je njen spol, ki ga, podobno kot pri ljudeh, določata kromosoma X in Y. Sorte navadne konoplje so lahko enodomne ali dvodomne. Dvodomne sorte so bolj primerne za pridobivanje vlaken, medtem ko so enodomne sorte primernejše za pridelavo semen ter za kombinirano uporabo (npr. seme in stebla) (Berenji in sod., 2013). Molekulsko določanje spola konoplje ima velik pomen in uporabo predvsem pri žlahtnjenju. S pomočjo molekulskih označevalcev (Mendel in sod., 2016) je mogoče spol določiti že pri zelo mladih rastlinah ali celo semenih. To je z vidika žlahtnjenja koristno predvsem zato, ker lahko izločimo nezaželene moške rastline, še preden bi lahko s pelodom oprašili ciljne ženske rastline, ki jih želimo uporabiti za križanja. Pri raziskavah smo najprej iz rastlinskega tkiva izolirali DNA, nato pa smo uporabili tri različne DNA označevalce (SCAR119, SCAR323 in MADC2) za določanje spola (Mendel in sod., 2016). Ugotovili smo, da ustrezne rezultate dobimo pri označevalcih SCAR119 (119 bp dolg pomnožek) in MADC2 (390 bp dolg pomnožek), medtem ko smo označevalec SCAR323 izločili zaradi pomnoževanja nespecifičnih pomnožkov pri ženskih in moških rastlinah. Najustreznejša temperatura prileganja začetnih oligonukleotidov je bila 52 °C. Preizkusili smo tudi določanje spola z direktnim PCR, pri katerem ni potrebno predhodno izolirati DNA iz tkiv. Za ta namen smo uporabili rastlinske liste na katerih smo preizkusili štiri različne PCR zmesi in različne temperature prileganja začetnih oligonukleotidov. Prileganje začetnih oligonukleoidov je bilo ponovno najboljše pri temperaturi 52 °C. Ugotovili smo, da dobimo enake rezultate pri molekulski analizi iz izolirane DNA ali s pomočjo optimiziranega protokola za direktni PCR. Prednost uporabe direktnega PCR je predvsem v hitrejšem postopku določanja spola, saj ni potrebno izolirati DNA.

Ključne besede: *Cannabis sativa* L., določanje spola, molekulski označevalci

ABSTRACT

A key feature associated with hemp (*Cannabis sativa* L.) cultivation and breeding is its sex. As with humans, it is determined by X and Y chromosomes. Hemp varieties are mostly dioecious, but they have been bred to be monoecious. Dioecious varieties are used mainly for fiber production while monoecious varieties are used for production of seeds or for both (seeds and fiber) (Berenji et al., 2013). Molecular determination of sex of hemp has great importance and is used primarily for breeding. With the help of molecular markers (Mendel et al., 2016), gender can be determined already in very young plants or even seeds. This is beneficial from the breeding point of view, mainly because it is possible to eliminate unwanted male plants before they pollinate the target female plants that are meant for crossings. DNA from plant tissue was isolated and tested by three different DNA markers (SCAR 119, SCAR 323 and MADC 2) for sex determination (Mendel et al., 2016).

Expected results were obtained only with markers SCAR119 (amplicon length 119 bp) and MADC2 (amplicon length 390 bp), while marker SCAR323 was discarded as inaccurate. The most suitable annealing temperature was 52 °C. Sex determination was also tested with direct PCR, for which it is not necessary to isolate DNA from plant tissues; only a small part of plant tissue (e.g., leaves) is crushed in a specific buffer. Four different protocols for the preparation of the PCR solution and different annealing temperatures were tested. The most suitable annealing temperature was again 52 °C. The same results were obtained with molecular analysis of isolated DNA and with direct PCR. The main advantage of using direct PCR is primarily time saving in the sex determination process, since it is not necessary to isolate DNA.

Key words: *Cannabis sativa* L., sex determination, molecular markers

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ATTEMPT TO INDUCE HAPLOIDS OF CANNABIS (*Cannabis sativa* L.) WITH *in vivo* GYNOGENESIS TECHNIUQE USING IRRADIATED POLLEN

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POVZETEK

Pri navadni konoplji prevladujejo populacijske sorte, saj pravih F1 hibridov zaradi težav pri pridobivanju čistih linij še ni. Poročamo o prvih poskusih pridobitve haploidnih rastlin konoplje, izzvanih s postopki *in vitro* ginogeneze, stimulirane s pelodom obsevanim z X-žarki. Močno obsevan pelod namreč lahko spodbudi razvoj embrija, s tem da se očetova garnitura kromosomov ne vključi. Izvedli smo serijo poskusov *in vitro* kalitve peloda konoplje treh sort. Preizkusili smo sestavo več gojišč za kalitev peloda in izbrali dve najugodnejši. Preizkusili smo več načinov obsevanja ter ugotovili, da je optimalen način obsevanje mešanice peloda tretiranega v posodici z zvišano vlažnostjo zraka. Testirali smo odmerke sevanja od 180 do 1326 Gy ter ugotovili, da je kalivost peloda po obsevanju upadala z večanjem doze obsevanja. Presenetljivo je bil pelod konoplje sposoben vsaj delne kalitve tudi pri najvišjem odmerku sevanja, kar kaže na veliko radiorezistenco. Z obsevanim pelodom smo opraševali ženske rastline štirih sort. Izvajamo serijo poskusov izolacije embrijev v različnih terminih po oprašitvi (od 7 do 14 dni) z izolacijo nezrelih embirjev na regeneracijsko gojišče. Uspešnost inokulacije je bila visoka, embriji pa se različno uspešno razvijajo. Oplojeni ovariji na rastlinah oprašenih z obsevanim pelodom, se razvijajo počasneje kot semena, ki so se razvijala na rastlinah oprašenih z neobsevanim pelodom. Preliminarni rezultati testiranja ploidnosti s pretočno citometrijo so pokazali diploidni status regenerantov, kar kaže, da pri teh serijah verjetno ni prišlo do eliminacije kromosomov. Poskuse nadaljujemo z drugimi termini inokulacije in odmerki sevanja.

Ključne besede: *Cannabis sativa* L., haploidi, ginogeneza, obsevanje, X-žarki

ABSTRACT

Due to inbreeding depression and flower characteristics it is difficult to obtain homozygous lines of cannabis. So far no F1 hybrid varieties exist, varieties are either open pollinated or synthetic. Our attempts were focused on protocols leading to gynogenic haploid induction stimulated with X-ray irradiated pollen. As shown in other species germination on stigma of heavily irradiated pollen can stimulate the development of embryos but without the father's chromosome set.

A series of experiments were performed with in vitro pollen germination of 3 cannabis varieties. The composition of several growth media were tested and 2 were chosen as most suitable. Several pollen irradiation treatments were tested and the optimal method was as follows: extracted pollen mixture was treated in a petri dish with increased air humidity. Doses varied from 180 to 1326 Gy, pollen germination decreased following irradiation with increasing radiation dose. Surprisingly, cannabis pollen was capable of at least partial germination even at the highest dose of radiation, indicating high radioresistence. Following pollination a series of embryos isolation was carried out 7 to 14 days after pollination. Isolated immature embryos were placed on the regeneration medium. Fertilized

ovaries on plants pollinated with irradiated pollen developed slower than seeds that were developed on plants pollinated by non-irradiated pollen. Preliminary results of ploidy test with flow cytometry showed the diploid status of regenerants. Experiments are continued with other terms of embryo extration and radiation doses.

Key words: *Cannabis sativa* L., haploids, gynogenesis, irradiation, X-ray

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TISSUE CULTURE OF HEMP (*Cannabis sativa* L.): MICROPROPAGATION AND ADVENTITIOUS REGENERATION

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IZVLEČEK

Mikropropagacija je tehnika hitrega klonskega razmnoževanja v *in vitro* razmerah, ki omogoča visoko frekvenco razmnoževanja rastlin. Adventivna regeneracija pa je tehnika regeneracije rastlin iz ne-meristematskega tkiva in nam omogoča uporabo genskega inženiringa za raziskovalne ali žlahtniteljske namene. V študiji smo želeli vzpostaviti protokole za sterilizacijo semen, vzpostavitev *in vitro* kulture poganjkov, mikropropagacijo poganjkov in za adventivno regeneracijo rastlin konoplje iz različnih rastlinskih izsečkov (kotiledonov in hipokotilov). Poleg tega smo želeli preizkusiti uporabnost ISSR markerjev za ugotavljanje pojava somaklonske variabilnosti. Izkazalo se je, da je tvorba poganjkov sorte Finola najboljša na gojišču z dodanim hormonom meta-Topolin, tvorba poganjkov pri sorti Fedora 17 pa je bila najboljša na MS gojišču s hormonom TDZ. Tvorba korenin je bila pri obeh sortah najboljša na MS gojišču brez dodanih hormonov. V poskusih adventivne regeneracije so se za najbolj regenerativno tkivo izkazali izsečki kotiledonov. S preizkušenimi ISSR markerji smo uspeli dokazati genetske razlike med rastlinami sorte Fedora 17, pojava somaklonske variabilnosti pa ne.

Ključne besede: *Cannabis sativa* L., mikropropagacija, adventivna regeneracija, ISSR markerji

ABSTRACT

Micropropagation is a technique of rapid *in vitro* clonal propagation of plants, which enables their high frequency multiplication. Adventitious regeneration is a process of *in vitro* production of new plant tissues and organs from non-meristematic cells and is used for genetic engineering in research and breeding. To establish both protocols, it is very important to test the regeneration ability of different types of explants. The aim of the study was to develop protocols for the sterilization of hemp seeds, the establishment of aseptic cultures, micropropagation, and adventitious regeneration from cotyledon and hypocotyl explants. The best medium for the formation of lateral shoots of the variety Finola was MS medium supplemented with the hormone meta-Topoline, while for the variety Fedora 17, a higher number of axillary shoots formed on MS medium supplemented with TDZ. Cotyledon explants proved to be more regenerative than hypocotyl explants. For both varieties, MS medium without hormones proved to be most suitable for rooting. Our results with inter-simple sequence repeats (ISSRs) showed genetic differences among plants of the variety Fedora 17, while they did not confirm the occurrence of somaclonal variation.

Key words: *Cannabis sativa* L., micropropagation, adventitious regeneration, ISSR markers

INTRODUCTION

There are only few research reports about tissue culture of hemp. Most of these studies aimed at developing cell culture systems for the production of secondary metabolites, particularly the THC class of cannabinoids that are characteristic of the genus *Cannabis* (Turner et al. 1980). Techniques of genetic engineering require the high frequency regeneration of plants, so it is very important to test the ability of tissue regeneration from different plant tissues and organs.

Micropropagation is a plant tissue culture method of rapid clonal propagation *in vitro.* It involves the culture of small sections of tissues and organs (explants) on defined aseptic culture media under controlled environmental conditions. This technique has many advantages over conventional vegetative propagation, since it enables the propagation of a high number of uniform and pathogen-free plants in a short time. The success of micropropagation depends on several factors, such as the composition of the culture medium, growth environment and genotype. The development of procedures may be of great commercial value for the cannabis industry (Wang et al. 2009; Kumar et al. 2011). In this regard, the establishment of an aseptic culture is an important step. With the proper selection of plant material sterilization, this process can make a major impact on the success of micropropagation. The goal of the treatment is to sterilize undesired microorganisms from the surface of seeds or other starting material, with minimal damage to the plant material.

Inter-simple sequence repeats (ISSRs) are regions in the genome flanked by microsatellite sequences. ISSR markers belong to a class of multilocus dominant genetic markers. They usually amplify multiple DNA fragments in a single reaction, since their primers anneal on a large number of loci across the genome of any species. There is therefore no need first to know the DNA sequences of the target regions. They are used for analysis of genetic variation of various organisms (Ng et al. 2015). ISSR markers are easy to use, low-cost and methodologically less demanding than other markers, making them ideal genetic markers for beginners and for organisms whose genetic information is lacking.

MATERIAL AND METHODS

Plant material. Seeds of hemp (*Cannabis sativa* L.) varieties Fedora 17, Finola and KC-Dora were obtained from the Agriculture Institute of Slovenia.

Sterilization. The hemp seeds were surface sterilized using six different methods:

1. Sterilization with 1.66 % dichloroisocyanuric acid (DICA) for 20 min.
2. Sterilization with 1.66 % DICA for 30 min.
3. Washing under running tap water for 15 min, followed by surface sterilization with 1.66 % DICA for 20 min.
4. Quick dipping in 70 % ethanol, followed by sterilization with 1.66 % DICA for 20 min.
5. Dipping in 70 % ethanol for 30 sec, followed by sterilization with 1.66 % DICA for 20 min.
6. Dipping in 70 % ethanol for 2 min, followed by sterilization with 1.66 % DICA for 20 min.

After sterilization, all treated seeds were rinsed with sterile doubled distilled water to remove traces of the sterilizing agents. The sterilized seeds were germinated on MS (Murashige and Skoog, 1962) medium with 30 g L^{-1} sucrose, 8 g L^{-1} agar, pH 5.8, and left to germinate at 25 °C under 16 h photoperiod.

Axillary buds and root induction. All the media for induction of axillary shoots and rooting contained MS basal medium, 30 g L^{-1} sucrose, 8 g L^{-1} agar and pH 5.8. Media for the induction of axillary shoots were composed of: 1. MS basal medium, 2. MS basal medium supplemented with 0.1 mg L^{-1} TDZ or 3. MS basal medium supplemented with 0.5 mg L^{-1} meta-Topoline. To choose the

optimal medium for root induction, the shoots were inoculated onto: 1. MS basal medium, 2. MS basal medium supplemented with 0.5 mg L^{-1} IBA and 500 mg L^{-1} activated charcoal or 3. MS basal medium supplemented with 0.5 mg L^{-1} meta-Topoline. The cultures were incubated at 25 °C with a 16 h photoperiod.

Adventitious regeneration. Plant explants (cotyledons, hypocotyls) were inoculated on nine different media based on the MS basal medium, supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} agar (pH 5.8) and the plant hormones presented in Table 1.

Table 1: Media used for adventitious regeneration.

Medium	TDZ (mg L^{-1})	NAA (mg L^{-1})
KON1	0.2	0.1
KON2	0.2	0.2
KON3	0.2	0.5
KON4	0.4	0.1
KON5	0.4	0.2
KON6	0.4	0.5
KON7	0.8	0.1
KON8	0.8	0.2
KON9	0.8	0.5

The cultures were kept at 21°C with a 16 h photoperiod.

ISSR markers. A total of eight primers (UBC_826, UBC_834, UBC_835, UBC_845, UBC_856, (GGGGT)_3M, (GGAT)_4H, (AAG)_6Y) were used in the ISSR analysis. DNA amplification was performed with an initial denaturation for 3 min at 95 °C, followed by 45 cycles of 30 sec at 95 °C, 30 sec at 50 °C, 3 min at 72 °C, and a final 7 min elongation step at 72 °C. All PCR reactions were performed with KAPA Taq DNA polymerase. Amplified bands were separated by electrophoresis on 2.0 % agarose gel and visualized with ethidium bromide.

RESULTS AND DISCUSSION

Sterilization. For both varieties, sterilization with DICA proved to be the most suitable, with germination rates of 77.5 % and 45 % for varieties Finola and KC Dora, respectively. All combinations of sterilization supplemented with 70 % ethanol were less appropriate, since they prevented germination of sterilized seeds. The results are presented in Table 2.

Table 2: Number of germinated and infected seeds of varieties Finola and KC-Dora three and six days after inoculation.

Variety	Sterilization	No of inoculated seeds	No of germinated seeds after 3 days (%)	No of infected seeds after 3 days (%)	No of germinated seeds after 6 days (%)	No of infected seeds after 6 days (%)
Finola	DICA 20 min	40	17 (42.5)	0 (0)	25 (62.5)	0 (0)
Finola	DICA 30 min	40	31 (77.5)	0 (0)	31 (77.5)	0 (0)
Finola	H_2O 15 min + DICA 20 min	40	21 (52.5)	0 (0)	29 (75.5)	0 (0)
Finola	quick ethanol + DICA 20 min	40	0 (0)	0 (0)	6 (15)	0 (0)
Finola	ethanol 30 sec + DICA 20 min	40	0 (0)	0 (0)	2 (5)	0 (0)
Finola	ethanol 2 min + DICA 20 min	40	1 (2.5)	0 (0)	4 (10)	0 (0)
KC Dora	DICA 20 min	40	18 (45)	9 (22.5)	18 (45)	24 (60)
KC Dora	DICA 30 min	40	15 (37.5)	23 (57.5)	15 (37.5)	31 (77.5)
KC Dora	H_2O 15 min + DICA 20 min	40	11 (27.5)	19 (47.5)	11 (27.5)	31 (77.5)
KC Dora	quick ethanol + DICA 20 min	40	0 (0)	20 (50)	2 (5)	29 (72.5)
KC Dora	ethanol 30 sec + DICA 20 min	40	0 (0)	10 (25)	0 (0)	18 (45)
KC Dora	ethanol 2 min + DICA 20 min	40	0 (0)	4 (10)	2 (5)	12 (30)

Axillary buds induction and root induction. The number and length of axillary shoots and roots obtained on different shoot induction media are presented in Tables 3 and 4.

Table 3: Average length and number of axillary shoots of varieties Fedora 17 and Finola obtained on different shoot induction media.

Variety	Medium	Number of inoculated nodes	Average length of axillary shoots (cm)	Number of axillary shoots
Fedora 17	MS medium	16	2.06	20
	MS + 0.1 mg L^{-1} TDZ	16	1.17	21
	MS + 0.5 mg L^{-1} m-Topoline	15	1.28	15
Finola	MS medium	16	2.93	27
	MS + 0.1 mg L^{-1} TDZ	16	1.28	30
	MS + 0.5 mg L^{-1} m-Topoline	16	2.06	33

For both hemp varieties tested (Fedora 17 and Finola), the longest axillary shoots developed on MS medium. A higher number of shoots of variety Fedora 17 developed on MS medium supplemented with hormone TDZ, while for Finola a higher number of developed shoots was obtained on MS medium with meta-Topoline. Some of the shoots also formed roots on these media, while the others were transferred to three different root induction media. The results are presented in Table 4.

Table 4: Number and average length of roots of varieties Fedora 17 and Finola.

Variety	Medium	No of inoculated shoots	No of developed roots	Average length of roots (cm)
Fedora 17	MS medium	5	10	3.19
	MS + 0.5 mg L^{-1} IBA + activated charcoal	14	0	/
	MS + 0.5 mg L^{-1} m-Topoline	8	3	0.67
Finola	MS medium	13	16	1.63
	MS + 0.5 mg L^{-1} IBA + activated charcoal	14	0	/
	MS + 0.5 mg L^{-1} m-Topoline	14	0	/

The highest number of roots and the longest roots developed on MS medium for both tested hemp varieties (Fedora 17 and Finola). The plants that did not form roots *in vitro* dried out during acclimatization. The results showed that *in vitro* rooting is essential for successful acclimatization of hemp plants, since none of the plants in our experiment formed roots *in vivo*.

Adventitious regeneration. Seventeen plantlets developed from the inoculation of cotyledon and hypocotyl explants on media KON1-KON9. Some of the explants formed roots, while all of them formed callus on the media tested. In order to stimulate callus cells to differentiate into shoots, callus was subcultured on three different shoot induction media: 1. MS medium, 2. MS medium supplemented with 0.1 mg L^{-1} TDZ and 3. MS medium with 0.5 mg L^{-1} meta-Topoline; only one plantlet regenerated from callus transferred onto MS medium.

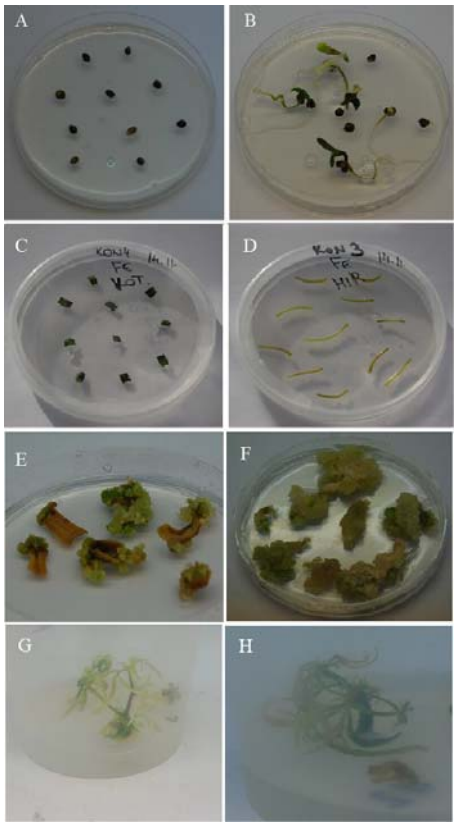

Figure 1: Steps of adventitious regeneration: inoculated seeds (A), germination of seeds (B), cotyledon explants (C, E), hypocotyl explants (D, F) and regenerated plantlets (G, H).

Acclimatization. The plants that formed roots on root induction media were transplanted into soil in acclimatization chambers. Only three (out of seventeen) plants were successfully acclimatized. All of them were rooted on MS medium without hormones and were propagated from the variety Fedora 17.

Figure 2: Successfully acclimatized micropropagated plants of the variety Fedora 17.

ISSR markers. Inter-simple sequence repeats are useful tools for checking somaclonal variation caused by adventitious regeneration. In our experiments, the amplified ISSR analysis was first performed on one DNA sample of hemp. The results showed that all eight ISSR markers amplified more than one locus, which makes them useful for the detection of mutations. Primers UBC_826 and (GGAT)_4H showed the highest number of amplified bands and were therefore used for further analyses.

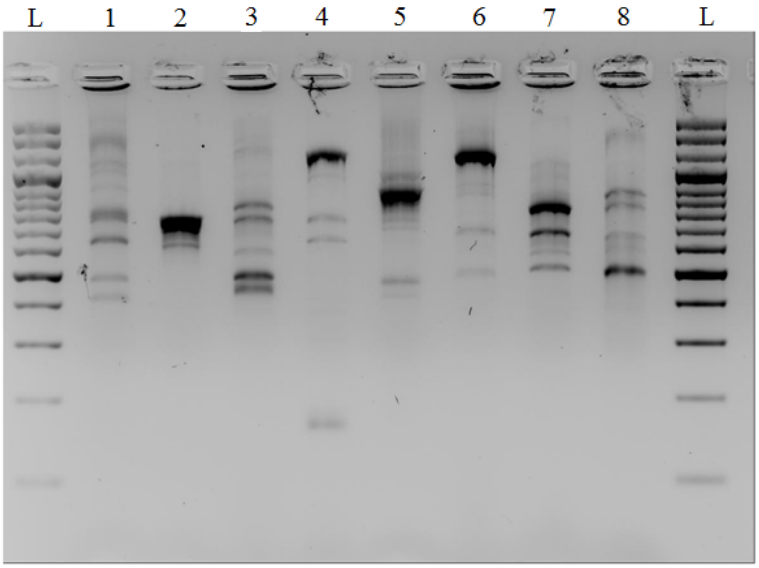

Figure 3: Inter-simple sequence repeats (ISSR) amplification pattern obtained with primers UBC_826 (1), UBC_834 (2), UBC_835 (3), UBC_845 (4), UBC_856 (5), (GGGGT)_3M (6), (GGAT)_4H (7), (AAG)_6Y (8).

Figure 4 shows the results of ISSR amplification of Fedora 17 with primer UBC_826. DNA was isolated from nine plants developed through adventitious regeneration, eight plants grown from seeds in a greenhouse and thirteen calluses.

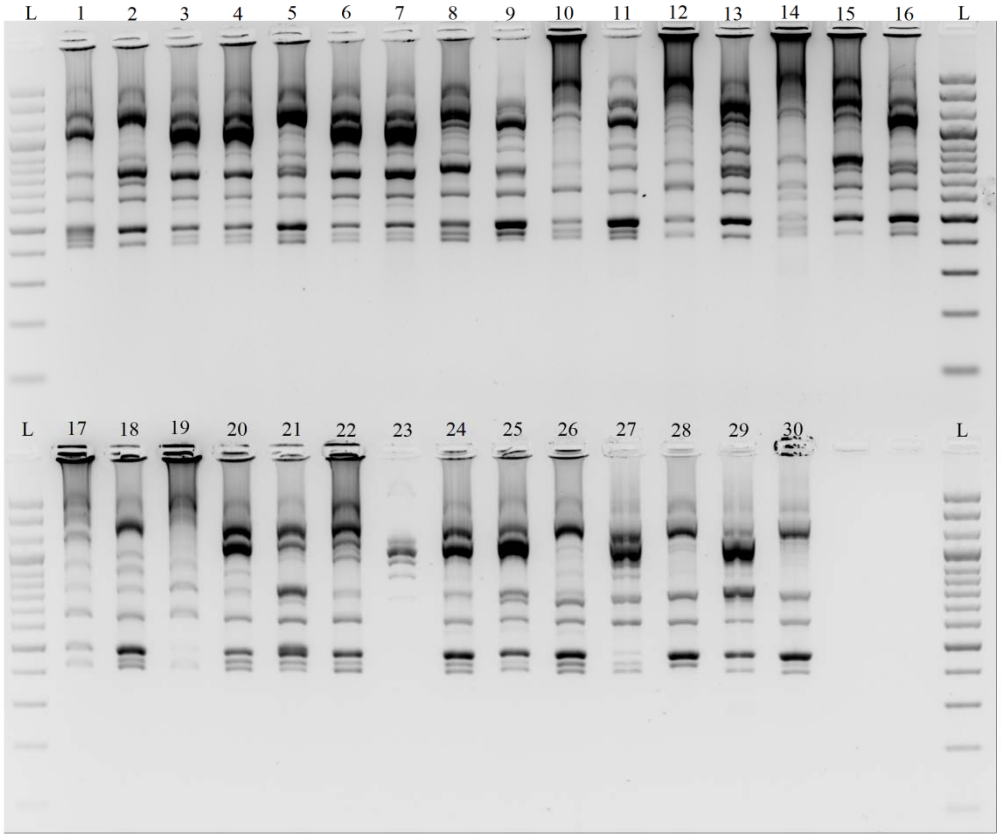

Figure 4: Amplification results obtained with ISSR marker UBC_826. DNA was extracted from in vitro regenerated shoots (1–9), from plants grown in a greenhouse (10–17) or from callus cells (18–30).

Although we could not identify genetic differences induced during *in vitro* culture, our results showed genetic differences among explants and proved the usefulness of ISSR markers for hemp genetic analysis.

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POSTERS – MOLECULAR BASIS OF DISEASES

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EXPRESSION OF DNA SENSORS AFTER IRRADIATION OF B16F10 MOUSE MELANOMA CELLS

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POVZETEK

Radioterapija deluje na celice citotoksišno preko poškodb DNA, hkrati pa povzroči spremembo tumorskega mikrookolja in posledično poveča aktivacijo imunskega sistema. Med drugim učinkovitost radioterapije temelji na aktivaciji receptorjev za zaznavo vzorcev, t.j. senzorjev DNA ter indukciji interferona tipa I (IFN). IFN sproščajo predvsem imunske celice, vendar jih lahko proizvajajo tudi tumorske celice. Naš cilj je bil ugotoviti ali obsevanje melanomskih celic B16F10 povzroči povečano izražanje nekaterih senzorjev DNA in ali to vodi v odziv IFNβ1.

Izražanje citosolnih senzorjev DNA (DDX41, DDX60, DHX9, DHX36, LRRFIP1, DAI/ZBP1, p204, cGAS in STING) smo preverili z metodo verižne reakcije s polimerazo v realnem času (qPCR) na nivoju mRNA v različnih časovnih točkah po obsevanju. Izražanje IFNβ1 smo izmerili na nivoju mRNA z analizo qPCR in na proteinskem nivoju z imunofluorescenčnim označevanjem.

Obsevanje tumorskih celic je povzročilo časovno in dozno odvisno povečano izražanje naslednjih citosolnih senzorjev DNA: DDX60, DAI/ZBP1, p204 ter tudi IFNβ1. Izražanje je bilo najbolj povečano 48 ur po obsevanju z dozami nad 4 Gy. Izražanje senzorja cGAS se po obsevanju ni spremenilo, senzorja STING pa je bilo povečano le 24 h po obsevanju z dozami nad 4 Gy.

Rezultati kažejo, da obsevanje melanomskih celic B16F10 lahko preko zaznavanja citosolne DNA sproži proizvodnjo IFN1β, kar prispeva k celični smrti po obsevanju.

Ključne besede: mišji melanom B16F10, citosolni senzorji DNA, obsevanje, interferon β1

ABSTRACT

Radiotherapy has a cytotoxic effect on cells through DNA damage. It can also alter the tumor microenvironment and consequently enhance activation of the immune system. Among other factors, efficiency of radiotherapy relies on the activation of pattern recognition receptors i.e. DNA sensors leading to a type I interferon (IFN) response. IFNs were reported to be produced mainly by immune cells, however they can also be produced by tumor cells. Our aim was to determine if irradiation causes upregulation of DNA sensors in B16F10 melanoma cells and whether this leads to IFNβ1 response.

The expression of several cytosolic DNA sensors (DDX41, DDX60, DHX9, DHX36, LRRFIP1, DAI/ZBP1, p204, cGAS and STING) was evaluated by quantitative polymerase chain reaction (q-PCR) analysis of purified mRNA at different time points after irradiation. The expression of IFNβ1 was

measured at the mRNA level by q-PCR and at the protein level by immunofluorescence labelling measured by flow cytometry.

Irradiation of tumor cells increased the expression of DNA sensors DDX60, DAI/ZBP1, p204 and of IFNβ1 in a time- and dose-dependent manner with the highest expression 48 h after irradiation with doses higher than 4 Gy. Expression of cGAS did not vary after irradiation, and expression of STING was upregulated only 24 h after irradiation with doses higher than 4 Gy.

These results indicate that irradiation can trigger the production of IFNβ1 by sensing of cytosolic DNA sensors in B16F10 melanoma cells, which can contribute to the cell death following irradiation.

Key words: mouse melanoma B16F10, cytosolic DNA sensors, irradiation, interferon β1

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INFLUENCE OF POLYMORPHISMS IN *TPMT* GENE AND GENES INVOLVED IN FOLATE AND METHIONINE METABOLISM ON MATERNAL AND FETAL MARKERS OF OXIDATIVE STRESS AND PREGNANCY OUTCOMES

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POVZETEK

Nosečnost je pomembno obdobje, ko je zagotavljanje ravnotežja med tvorbo in odstranjevanjem reaktivnih kisikovih zvrsti zelo pomembno. Oksidativni stres (OS) med nosečnostjo lahko vpliva na rast in razvoj ploda. BHMT, GNMT, MTRR, MTHFD1 in MTHFR so ključni encimi metioninskega in folatnega cikla ter so vključeni v regeneracijo metionina, sintezo cisteina in glutationa ter v biosintezo S-adenozil-metionina (SAM). SAM je glavni donor metilne skupine v reakcijah transmetilacije in je stabilizacijski kofaktor tiopurin S-metiltransferaze (TPMT). TPMT je v farmakogenomiki tiopurinov dobro poznan encim, čeprav njegova endogena funkcija še ni znana. TPMT bi lahko bil vpleten v detoksifikacijske procese in v obrambni antioksidantni odziv. V tej študiji smo preiskovali povezavo med genotipi *TPMT*, *MTHFR*, *BHMT*, *GNMT*, *MTRR* in *MTHFD1* in označevalci OS v maternalnem in fetalnem okolju v 2. trimesečju nosečnosti ter izidi nosečnosti pri 147 preiskovankah. Označevalce OS smo izmerili v eritrocitih matere, materinem urinu in v amnijski tekočini. Po rojstvu smo odčitali oceno po Apgarjevi, gestacijsko starost, težo in dolžino novorojenčka ter značilnosti posteljice. Aktivnost superoksid dismutaze (SOD) v materinih eritrocitih je bila višja pri preiskovankah, ki so nosile mutirane alele *TPMT*. Še bolj izrazito povečanje aktivnosti SOD je bilo prisotno, ko smo poleg mutiranih alelov *TPMT* v analizo vključili še genotipe *MTHFR* z znižano encimsko aktivnostjo. Novorojenčki, katerih matere so imele sočasno prisotne mutirane alele *TPMT* in *MTHFR*, so imeli pogosteje prisotno oceno po Apgarjevi 7 ali manj. Otroci preiskovank z nemutiranim genotipom *MTHFR* so ob rojstvu dosegli večjo gestacijsko starost. Naše ugotovitve kažejo na možno vlogo *TPMT* pri odzivu na OS.

Ključne besede: nosečnost, oksidativni stres, TPMT, folatni cikel

ABSTRACT

The perinatal period is a critical time for maintaining a balance between the production and detoxification of reactive oxygen species. Oxidative stress (OS) during pregnancy can have a profound and sustained impact on the development of the fetus. BHMT, GNMT, MTRR, MTHFD1 and MTHFR are the key enzymes of methionine and folate cycle and are involved in the regeneration of methionine, synthesis of cysteine and antioxidant glutathione and in biosynthesis of S-adenosyl-methionine (SAM). SAM is the main methyl donor in transmethylation reactions and a stabilizing cofactor of thiopurine S-methyltransferase (TPMT). TPMT is well known pharmacogenomics entity, although its endogenous function is still unknown. There are some suggestions that TPMT might be

involved in the processes of detoxification and OS response. In this study the correlation of common *TPMT*, *MTHFR*, *BHMT*, *GNMT*, *MTRR* and *MTHFD1* genotypes of 147 pregnant women with markers of OS in maternal and fetal compartments during the 2nd trimester of pregnancy, as well as pregnancy outcomes, was investigated. Markers of OS were measured in maternal erythrocytes, maternal urine and in amniotic fluid. After the delivery, Apgar score, gestational age at delivery, birthweight, birthlength and characteristics of the placenta were noted. Superoxide dismutase (SOD) activity in maternal erythrocytes was higher in women carrying variant *TPMT* alleles. Even more prominent increase in SOD activity was indicated when in addition to variant *TPMT*, enzyme activity lowering MTHFR genotypes were included. Infants of mothers with concomitant presence of variant TPMT and MTHFR genotypes had higher frequency of Apgar scores 7 and less. Infants of mothers with wild-type MTHFR genotypes tended to reach higher gestational age at delivery. Our findings indicate that TPMT might play a role in the OS response.

Key words: Pregnancy, oxidative stress, TPMT, folate cycle

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ANALYSIS OF POLYMORPHISMS rs10738760 AND rs6921438 IN TYPE 2 DIABETIC PATIENTS WITH PROLIFERATIVE DIABETIC RETINOPATHY OF SLOVENE ORIGIN

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IZVLEČEK

Diabetična retinopatija (DR) je bolezen, ki se pojavi kot posledica slabo urejene sladkorne bolezni. Poznamo dve obliki bolezni: neproliferativno diabetično retinopatijo (NPDR) in proliferativno diabetično retinopatijo (PDR). Gen *VEGF* uravnava nastanek novih žil v endoteliju, njegovo nekontrolirano izražanje pa lahko privede do bolezenskih stanj kot je PDR. Raven VEGF je pod vplivom številnih dejavnikov, med drugim polimorfizmov rs10738760 in rs6921438. Analizirali smo vpletenost teh dveh polimorfizmomov na razvoj PDR. V raziskavo smo vključili 505 bolnikov s sladkorno boleznijo tipa 2 in jih razdelili v dve skupini: skupino z PDR (143 bolnikov) in v kontrolno skupino (362 bolnikov). Kontrolna skupina je vključevala bolnike ki imajo sladkorno bolezen tipa 2 več kot 10 let in nimajo PDR ali pa imajo blago obliko DR. Genotipizacijo polimorfizmov smo izvedli z metodo PCR v realnem času. Izvedli smo tudi sistematični pregled literature o povezavi polimorfizmov rs10738760 in rs6921438 z DR. Rezultati naše raziskave kažejo, da povezave med rs10738760 in rs6921438 pri bolnikih s SB2 z PDR v Slovenski populaciji ni. Obstajajo še drugi polimorfizmi v genu *VEGF* ali polimorfizmi povezani z ravnijo cirkulirajočega *VEGF*, ki bi jih v prihodnje bilo smiselno v povezavi z diabetično retinopatijo še podrobneje raziskati.

Ključne besede: diabetična retinopatija, *VEGF*, polimorfizmi

ABSTRACT

Diabetic retinopathy (DR) is a disease that occurs after long lasting and poorly handled diabetes. Two forms of disease are known: nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). *VEGF* gene regulates development of new vessels in endothelium and increased expression can lead to many disease forms such as PDR. The VEGF levels are under control of several factors, including polymorphisms rs10738760 and rs6921438. In the present study we analyzed the involvement of these two polymorphisms with development of PDR. We analyzed 505 T2D patients and divided them in two groups: PDR group (n = 143) and control group (n = 362). Control group included patients that had T2D more than ten years and do not have PDR or have mild form of DR (NPDR). Genotyping of polymorphisms was performed using real-time PCR. Additionally, systematic literature search on rs10738760 and rs6921438 polymorphisms and DR was performed. Results of this study show that there is no association between rs10738760 and rs6921438 and T2D in Slovene patients with PDR. Several polymorphisms located within VEGF gene

or associated circulating VEGF levels are known, that should be researched on in the future, in association with diabetic retinopathy.

Key words: diabetic retinopathy, *VEGF*, polymorphisms

INTRODUCTION

Diabetic retinopathy (DR) is still the leading cause of vision loss in America and Europe in patients aged 20 to 74 years, and is one of many diabetic complications (Hampton et al, 2015). Almost all of the type 1 diabetics (T1D) get diagnosed with diabetic retinopathy in 15 years from diabetes diagnose, and >60% of T2D patients. The risk for developing DR can be reduced if early detected, maintaining low hypertension and good glycemic control. Clinically DR differs into two subgroups. One is nonproliferative diabetic retinopathy (NPDR) of which main characteristics are usually microaneurysms, hemorrhagy, wool spots and hard exudates that can be seen in eye background. NPDR is divided into three subgroups: mild, moderate and severe. Other DR subgroup is proliferative retinopathy (PDR) which occurs when new vessels start to form in eye background. PDR can also lead to macular edema and in major cases it can lead to vision loss (Tarr et al., 2013). Commercial treatments are already used such as anti-VEGF injections and laser treatment for NPDR and vitrectomy at the end stage of PDR.

Vascular endothelial growth factor (*VEGF*) is one of the most researched genes in association with DR, diabetes, nephropathy, and cancer. It is located on chromosome 6 and consists of 8 exons. Expression of the *VEGF* gene is important at the beginning of DR occurrence and its development. It advances microaneurysm development and vessel permeability. The main cause of *VEGF* expression is occurrence of hypoxia. Lower concentrations of oxygen allow expression of transcription factor *HIF1* (hypoxia inducible factor 1), which stimulates *VEGF* gene expression. Many *VEGF* polymorphisms for diabetic retinopathy development have been identified. The following polymorphisms have been most frequently associated with DR/PDR: rs833061, rs2010963, rs699947 and rs2010963. GWAS study by Debette et al. (2011) described two polymorphisms rs10738760 and rs6921438 which are not placed inside *VEGF* gene, to have a very large effect on circulating VEGF levels. Polymorphism rs6921438 is located on chromosome 6p21.1, close to *C6orf223* gene and 171 kb downstream of the *VEGF* gene. SNP rs10738760 on chromosome 9p24.2 is located between very low density lipoprotein receptor (*VLDLR*) and potassium voltage-gated channel subfamily V member 2 (*KCNV2)* genes. G allele of rs6921438 polymorphism was found to be associated with T2D and higher value of HbA1c levels in French population (Bonnefond et al., 2013). In the present study we tested the association between those two polymorphisms and PDR in Slovene population.

MATERIALS AND METHODS

Ethic statement. Patients included in the study, were acquainted with protocol and purpose of study. A written informed consent was obtained from every participant before collecting blood samples for molecular-genetic testing. The study was approved by Commission for Medicine Ethics by Ministry for health in Republic of Slovenia at regular session on 23th of March 2010.

Study design. A retrospective cross-sectional association study was performed to test an association between two candidate polymorphisms and PDR. A total of 505 T2DM Slovenian non related PDR patients were enrolled in the study. Family and personal anamnesis and clinical data for diabetics with and without DR were summarized by uniform questionnaire. Patients were included in the study at the University Eye Clinics Ljubljana and at the internist clinics (cardiologic, diabetic,

endocrinological) of the University Clinical Center in Ljubljana and Maribor. Fundus examination was performed by a senior ophthalmologist (MGP) after pupil dilatation (tropicamide and phenylephrine 2.5%) using slit-lamp biomicroscopy with non-contact lens, and was electronically documented with a 50°-angle fundus camera (Topcon-TRC 40-IX; Tokyo, Japan). The stage of diabetic retinopathy was determined according to the Early Treatment Diabetic Retinopathy Study Research Group retinopathy severity scale. Diabetics were divided according to the presence/absence of microvascular complication in two groups: PDR group (n = 143) and control group (n = 362). Control group consisted of T2DM patients with more than 10 years of diabetes duration and no signs of PDR or have mild form of DR. Characteristics of patients are represented in Table 1.

Table 1: Clinical and laboratory characteristics of patients with PDR and controls.

Characteristics	Cases	Controls	P value
Number	143	362	
Age (years)	63.4±9.5	64.7±10.2	0.2
Male sex (%)	70 (49.0)	161 (44.6)	0.4
Duration of diabetes (years)	18.8±6.2	11.8± 2.6	<0.001
Patients on insulin therapy (%)	104 (72.9)	174 (48.1)	<0.001
Systolic blood pressure (mm Hg)	143.9±23.1	143.1±18.4	0.7
Diastolic blood pressure (mm Hg)	83.8±10.2	83.5±10.1	0.8
BMI (kg/m^2)	28.4±4.6	30.1±5.3	<0.001
Total cholesterol (mmol/l)	5.1±1.2	4.9±1.1	0.09
LDL cholesterol (mmol/l)	3.1±1.0	2.7±0.9	0.001
HDL cholesterol (mmol/l)	1.1±0.3	1.2±0.3	0.001
Triglycerides (mmol/l)	2.3±1.3	2.4±1.8	0.3
HbA_{1c} (%)*	8.0±1.4	7.8±1.4	0.3

The values represent mean ± standard deviation. *The average value for haemoglobin A_{1C} (HbA_{1c}).

DNA isolation. DNA was isolated from peripheral blood using DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany).

Real-time PCR. Genotyping of polymorphisms was performed using TaqMan SNP Genotyping assay (Applied Biosystems, Foster City, CA, USA). We were following the manufacturer protocol for preparation of reaction mixture for both polymorphisms. Step 1. (Pre-PCR Read) represents reading of initial fluorescence; Step 2. (Cycling Stage) represents multiplication of sections, which includes pre-denaturation and denaturation at 95°C and alignment of specific initial oligonucleotides and probes at 60°C; Step 3. (Post – PCR Reads) reading of end fluorescence at 60°C. We have always applied minimum of three control samples of known genotype (homozygote sample for one allele, heterozygote sample and homozygote sample for other allele) and negative control.

Statistical Analysis. We have analyzed the allele discrimination by using StepOne Software version 2.2 (Applied Biosystems, Foster City, CA, USA).

RESULTS AND DISCUSSION

The results of the association study show no association with polymorphisms rs10738760, rs6921438 and PDR in Slovene population (Table 2). None of the genotypes in both polymorphisms have shown significant rise in certain tested group, neither in cases nor controls. Even though we expected significant rise of two polymorphisms in cases with PDR, no difference between cases and controls has been observed on polymorphisms genotype level.

Table 2: Adjusted p values for duration of diabetes, patients on insulin therapy, BMI, LDL and HDL cholesterol.

Inheritance Model	Genotype	Cases (143)	Controls (362)	Adjusted OR, 95 % CI/p-value
rs10738760	GG	30 (21.0)	89 (24.6)	Reference
Co-dominant	GA	81 (56.6)	186 (51.4)	0.89 (0.50–1.63)/0.7
	AA	32 (22.4)	87 (24.0)	1.08 (0.52–2.23)/0.8
rs6921438	GG	40 (28.0)	90 (24.9)	Reference
Co-dominant	GA	63 (44.0)	183 (50.5)	1.70 (1.00–2.86)/0.05
	AA	40 (28.0)	89 (24.6)	1.12 (0.64–1.98)/0.7

There are many polymorphisms of the *VEGF* gene associated with DR. Its polymorphisms also contribute to other diseases such as depression and cholesterol levels (Table 3). Understanding its expression and regulation may contribute to developing new treatments and a different perspective on disease understanding. Additionally polymorphisms located outside of the *VEGF* gene regions have been shown to affect circulating VEGF levels.

Table 3: A review of the literature testing the association between polymorphisms rs10738760 and rs6921438 with different diseases.

Polymorphism (allele)	Association	Reference
rs6921438	Circulating VEGF levels	Debette et al., 2011
rs6921438 (G)	Associated with T2D in French population	Bonnefond et al., 2013
rs6921438 (G)	Increased HbA1c levels in French population	Bonnefond et al., 2013
rs6921438	Not associated with DR in French, Danish population	Bonnefond et al., 2013
rs10738760	Not associated with T2D or DR in French, Danish population	Bonnefond et al., 2013
rs6921438	HDL-C and LDL-C plasma levels	Stathopoulou et al., 2012
rs6921438	Not associated with major depression in Spanish Caucasian population	Xie et al., 2017

DR (diabetic retinopathy), T2D (type 2 diabetes), HbA1c (glycated haemoglobin)

GWAS study from 2011 showed that the rs10738760 and rs6921438 polymorphisms explained nearly half of the variance in circulating *VEGF* levels (Debette et al., 2011). There has been strong association between G-allele of rs6921438 polymorphism and T2D risk in French but not in Dane population. Alongside the same allele has been found associated with increased HbA1c levels in French general population sample, but also not in Dane population. The G allele of rs6921438

polymorphism is the allele that is increasing VEGF levels in general population (Debette et al., 2011). Polymorphism rs10738760 was not associated with T2D in French nor Danish population. The two polymorphisms have also not been associated with macular edema nor DR or any other microvascular complications (Bonnefond et al., 2013). The study by Stathopoulou et al. (2012) reported association between SNP rs6921438 with HDL-C and LDL-C plasma levels. It has also been shown that rs6921438 could have negative effect in the cardiovascular system through decrease in HDL-C and increase in LDL-C levels and a decrease in VEGF levels. Xie et al. (2017) tested the association between rs6921438 SNP and major depression, however they did not find the association.

Despite the fact that there is no association between rs10738760 and rs6921438 polymorphisms and PDR in Slovene population, further research on those polymorphisms in different populations might contribute to new understanding of DR and other diseases. In future it is necessary to test other *VEGF* polymorphisms and polymorphisms affecting VEGF levels, previously found to be associated with the disease. Knowing the fact that the researched polymorphisms are known to explain nearly half of the variance in circulating *VEGF* levels, and because the VEGF itself is the most prominent protein for diabetic complications and other diseases development, it should be researched further.

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EXPRESSION OF DNA SENSORS AFTER ELECTROTRANSFER OF pDNA IN SPHEROIDS OF MOUSE MELANOMA CELL LINE B16F10

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POVZETEK

Prisotnost DNA v citosolu lahko aktivira DNA senzorje, kar sproži zaporedje reakcij znotrajceličnega signaliziranja, ki vodijo v prepis genov za vnetne citokine in kemokine. Poleg prepisovanja genov lahko prisotnost citosolne DNA vodi tudi do celične smrti. V predhodnih študijah smo že dokazali povečano izražanje senzorjev DNA na 2D celičnih kulturah mišjega melanoma B16F10. Tokrat smo raziskavo razširili in nadgradili s preučevanjem 3D modelov sferoidov, in tako vključiti vpliv molekularnih, biokemijskih in fizioloških značilnosti *in vivo* okolja na izražanje DNA senorjev.

Na ploščo s 96 vdolbinicami smo v 150 µl medija obogatenega s hidroksipropilmetil celulozo nasadili 300 melanoskih celic. Ko so sferoidi dosegli velikost 300 µm, smo izvedli elektroprenos s pDNA brez terapevtskega gena ali elektroprenos s pDNA z zapisom za zeleni fluorescentni protein (pEGFP1). Uspešnost transfekcije in mehanizme celične smrti smo preverili z uporabo pretočnega citometra, rast z merjenjem površine sferoida z uporabo mikroskopa., povečano izražanje senzorjev DNA pa z verižno reakcijo polimeraze v realnem času (qPCR).

Po elektroprenosu plazmida pEGFP1, je bilo transfeciranih približno 2% vseh celic sferoida. Rast sferoidov se je po elektroprenosu pDNA zmanjšala, a ni bila statistično različna kot po uporabi samih pulzov. Zaznali smo povečano izražanje enakih senzorjev DNA (DDX60, DAI, p204), kot pri 2D celični kulturi . Celice znotraj sferoida so po elektroprenosu s pDNA umirale z apoptozo.

Naši poskusi na sferoidih se skladajo z rezultati pridobljenimi na 2D celičnih kulturah in tumorjih mišjega melanoma. Tako lahko potrdimo, da so sferoidi pomemben povezovalni člen med *in vitro* in *in vivo* eksperimenti.

Ključne besede: elektroprenos, citosolni senzorji DNA, sferoidi, mišji melanom B16F10

ABSTRACT

DNA in the cytosol can activate cytosolic DNA sensors and consequently lead to upregulation of proinflammatory cytokines, chemokines and to cell death. We have previously shown increased expression of DNA sensors in adherent B16F10 melanoma cells afterelectrotransfer of plasmid DNA (pDNA). In this study, we expand our research to spheroids to represent the influence of molecular, biochemical and physiological features of the corresponding tissue *in vivo* on the expression of DNA sensors.

Melanoma cells (300 cells) were plated in each well of 96-well U-bottom plates in 150 µl growth medium supplemented with hydroxypropylmethyl cellulose. Electrotransfer of pDNA without therapeutic gene or coding for green fluorescent protein was performed when spheroids reached 300 µm in diameter. Transfection efficiency and types of cell death were determined by flow cytometry, growth by measuring the area of spheroid using microscopy, and expression of DNA sensor mRNAs by reverse transcription followed by quantitative PCR.

Flow cytometry analysis showed that 2% of cells in the spheroid are transfected. The growth of spheroids was reduced after electrotransfer of pDNA to the same level as after electroporation alone. mRNA levels of the DNA sensors DDX60, DAI, p204, which were increased in 2D cell cultures, was also increased in spheroids. The main cell death mechanism after pDNA electrotransfer was apoptosis, which confirmed the previous in vitro cell experiments.

To conclude, experiments on spheroids confirmed our previous findings in murine melanoma cell cultures, therefore spheroids are a promising tool for mimicking the tumor cells fraction of tumor microenvironment.

Key words: electrotransfer, cytosolic DNA sensors, spheroids, mouse melanoma B16F10

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IDENTIFICATION OF SEQUENCE VARIANTS ASSOCIATED WITH FAMILIAL ERYTHROCYTOSIS IN *EPO, EPOR*, *EPAS1* AND *VHL* GENES: FROM *IN SILICO* ANALYSIS TO CLINICAL RESEARCH

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IZVLEČEK

Redko bolezen eritrocitozo opredeljuje povečano število rdečih krvnih celic, povečan hemoglobin in hematokrit. Hormon eritropoetin (EPO) je preko svojega receptorja EPOR glavni regulator pri proizvodnji rdečih krvnih celic v kostnem mozgu. Gen endotelne PAS domene (*EPAS1)* se aktivira ob nizki vsebnosti kisika in sproži transkripcijo eritropoetina. V pogojih z normalno količino kisika se EPAS1 razgradi z Von Hippel-Lindau tumor supresor (VHL) proteinom. Policitemija vera (PV) je najpogostejša oblika primarne eritrocitoze, zaradi pridobljene mutacije v Janus kinaza genu (*JAK2)*. Primarne in sekundarne pridobljene oblike družinske eritrocitoze (DE) so povezane z različicami zaporedja v različnih genih, vključno z *EPOR*, *EPO*, *VHL* in *EPAS1*. Zaradi heterogenega genetskega ozadja, so potrebni dodatni diagnostični testi za opredelitev vzrokov pri slovenskih bolnikih z eritrocitozo. Nedavno identificirane različice *EPO* gena povezane z DE, so trenutno v procesu vključevanja v diagnostične postopke v Sloveniji. V naši študiji smo izvedli mutacijsko analizo za gena *VHL* in *EPAS1,* kot podlago za nov molekularno-genetski test pri *JAK2* negativni eritrocitozi. Sekvencirali smo tri eksone *VHL* gena in en ekson *EPAS1* gena. Prvotni rezultati niso pokazali različic zaporedja DNA, vendar je sekvenciranje dodatnih regij *EPAS1* in *VHL* gena v teku.

Ključne besede: eritrocitoza, različica zaporedja DNA, EPO regulacija

ABSTRACT

Rare haematological disorder erythrocytosis is defined by increased red blood cell number, haemoglobin and haematocrit. Hormone erythropoietin (EPO) is the main regulator of red blood cell production via its receptor EPOR in bone marrow. Erythropoietin transcription is activated by endothelial PAS domain (EPAS1) gene in low-oxygen conditions. In normoxia, EPAS1 is rapidly degraded by Von Hippel-Lindau tumor suppressor (VHL) protein. Polycythemia vera (PV) is the most common primary erythrocytosis due to acquired mutation in Janus kinase 2 (*JAK2*). Primary and secondary type of familial erythrocytosis (FE) is associated with inherited variants in various genes, including *EPOR*, *EPO*, *VHL* and *EPAS1*. Due to heterogenous genetic background, additional diagnostic tests to characterize causes in Slovenian patients with erythrocytosis are needed. Recently, identified sequence variants of the *EPO* gene associated with FE are in the process of integration into diagnostic procedures in Slovenia. In our study we performed mutational analysis for *VHL* and *EPAS1* genes as the basis for novel molecular-genetic test in *JAK2* negative erythrocytosis. We sequenced

three exons of *VHL* gene and one exon of *EPAS1* gene. In this preliminary study we did not identify polymorphisms. The study is in progress and undergoing sequencing of additional gene regions.

Key words: erythrocytosis, sequence variants, EPO regulation pathway

INTRODUCTION

Erythrocytosis is a heterogeneous group of disorders, caused by increased red blood cells (RBC) production resulting in elevated level of both, haemoglobin (Hb) and haematocrit (Ht)[1]. Production of RBC is tightly regulated by changes in oxygen tension, mainly through hormone erythropoietin (EPO) and its receptor EPOR. Expression of *EPO* gene is regulated by hypoxia-inducible factors (HIFs)[2,3]. Protein EPAS1 (endothelial PAS domain-containing protein 1, also known as HIF2A) is the main EPO regulator. Under normoxic conditions (21% O_2), VHL-complex binds to EPAS1, leading to ubiquitination and subsequently EPAS1 degradation (Figure 1). On contrary, in hypoxia due to low oxygen conditions (e.g. 1% O_2) association of EPAS1 with VHL protein is prohibited. Consequently, EPAS1 accumulates and forms a stable dimer complex with beta subunit (HIF1B) , which activates a number of genes, including *EPO*[2].

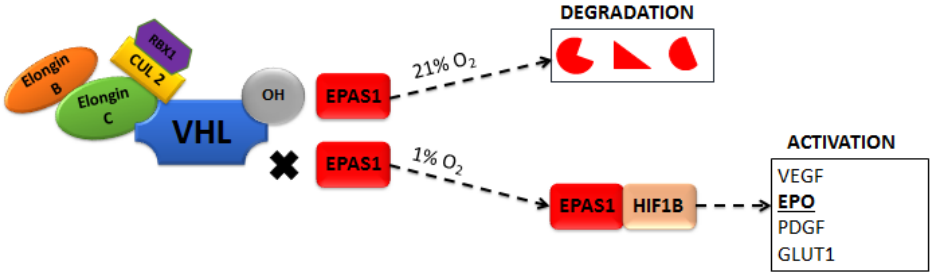

Figure 1: Oxygen sensing by VHL complex. In normoxia (21% O_2) EPAS1 is degraded, while in hypoxia (1% O_2) EPAS1, through HIF complex, promotes the expression of genes that mediate the cellular response to hypoxic conditions

Familial erythrocytosis (FE) is a type of erythrocytosis with various genes variants. Primary FE is associated with variants in *EPOR*, designated as familial erythrocytosis type 1 (ECYT1; OMIM #133100). Variants in globin genes and genes involved in oxygen-sensing pathway are a causes for secondary type of FE[4, 5]. *VHL* variants are causative for familial erythrocytosis type 2 (ECYT2; OMIM #263400), while *EPAS1* variants for familial erythrocytosis type 4 (ECYT4; OMIM #611783). Due to heterogenous genetic background of FE, patients need to be screened for sequence variants in various genes, with still many unidentified cause[4]. In order to identify cause for erythrocytosis symptoms in Slovenian patients with FE, researchers are establishing diagnostic tests for genes with known association to FE. In previous studies, two patients from Slovenian family with *JAK2* negative erythrocytosis (exclusion of PV) were screened for sequence variants in *EPO* and *EPOR* genes. Variants of the *EPO* gene associated with FE were identified and are currently in the process of integration into diagnostic procedures for FE in Slovenia[15].

The aim of our study was to review past analysis on *EPO* and *EPOR* genes and to extend diagnostic procedures and develop additional diagnostic tests for *VHL* and *EPAS1* genes variants. First, we reviewed the literature and online databases for EPAS1 and VHL variants associated with

erythrocytosis. In second part we analysed patients with FE and unaffected controls for sequence variants in selected regions of *VHL* and *EPAS1* genes.

MATERIAL AND METHODS

Subjects and healthy controls. The study was conducted on four patients with idiopathic erythrocytosis (Figure 2). All patients have elevated RBC, Ht and Hb, normal level of serum EPO and are negative for mutations in *JAK2* (exclusion of PV). For reference sequence, two unaffected controls were included in the study; a member of affected family (C3) and healthy volunteer with normal RBC, Ht and Hb (designated C1). The study was approved by local Ethical Committee (NME 115/08/15) and all subjects gave their informed consent.

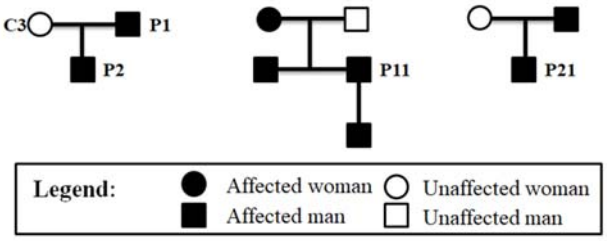

Figure 2: Family tree of families with FE involved in study. Legend: P - patients, C - healthy controls.

***In silico* analysis of *EPAS1* and *VHL* structure and variants associated with erythrocytosis.** Information about *EPAS1* and *VHL* sequence and variants in these genes were searched using genetic browser Ensembl release and dbSNP database on NCBI. Based on the literature search using VHL, EPAS1, hypoxia inducible factor 2, HIF2A, familial erythrocytosis, polymorphisms, sequence variants, elevated red blood cell, elevated hematocrit and elevated haemoglobin key words, we obtained publications from PubMed, UniProt and GeneCards to acquire previously reported associations between *VHL* or *EPAS1* gene and inherited erythrocytosis. Structure of VHL and interactions sites were extracted from RCSB PDB, Nextprot, VHLdb, BioGrid and published literature. *VHL* variants were aligned to genomic regions of previously reported interaction sites, which enabled to determine the effect on the function.

Isolation of DNA. Total leucocytes were isolated from peripheral blood. Genomic DNA was extracted from 1–2 10^7 cells by QIAamp DNA mini kit (Invitrogen). Total cellular RNA was isolated from 1–2 10^7 cells with Trizol (Invitrogen). Complementary DNA (cDNA) synthesis was performed with SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen).

Mutational analysis. Part of *EPOR* gene (exon 8), *EPO* (promotor, enhancer), *EPAS1* (exon 9, 12 and 16) and *VHL* (exons 1, 2 and 3) were amplified from cDNA or genomic DNA, using polymerase-chain-reaction (PCR) assay with KAPA HiFi HotStart PCR Kit (KapaBiosystems). Primers for gene *VHL* were extracted from publication[6], for *EPAS1* collected from Primer Designer™ Tool and for *EPO and EPOR* designed using PRIMERBlast. PCR products were cleaned with ExoSAP-IT (Affymetrix) or QIAquick PCR Purification Kit (Qiagene) and sequenced with Sanger DNA sequencing method (GATCBiotech). Sequences were analysed with Chromas (Technelysium) and aligned with reference sequence using "Align Sequences Nucleotide BLAST"[7]. Reference sequences: HIF2A (RefseqGene with ID number NG_016000.1) and VHL (RefseqGene with ID number NG_008212.3).

RESULTS AND DISCUSSION

***VHL* sequence structure and variants.** Based on the current version of Ensembl browser transcript VHL-201 has 2003 sequence variants. Literature review revealed positive association between ECYT2 and 20 sequence variants, which are spread throughout the whole protein (Figure 3)[1, 4, 6, 8–10]. Literature review showed four important interacting sites on VHL gen that are important for VCB-Cul2 complex formation (Figure 3). Variants on BC box (comprising amino acids 157–170) and Cul box (comprising amino acids 175–190) could disrupt formation of complex[11]. From residue 91 to 113 VHL is interacting with target protein HIF[12]. Interacting site from 100–155 was found to interact with chaperons Hsp70 and Tric (TCP-1 Ring Complex, also called CCT), which role is correctly biding of target protein VHL[13]. Out of twenty variants, eleven are located in the validated interacting sites of studied proteins. Variants positioned out of interacting sites could also have phenotypic effect, since there are cases of FE with variants out of interacting site. Those can still alter the strict structural requirements to cause loss of VHL function[14]. Genotypes of patients extracted from diverse, since disease can be caused by variant only in a single VHL allele, in both VHL alleles on the studies are same locus or when combined with changes on two different locus alleles. Eight possible genotypes were found to have a variation only in one allele (heterozygous SNV genotype), five recorded genotypes were found to have the same variant on both alleles (homozygous SNV genotype) and eleven possible genotypes have two different variants on two locus (compound heterozygous SNV genotype).

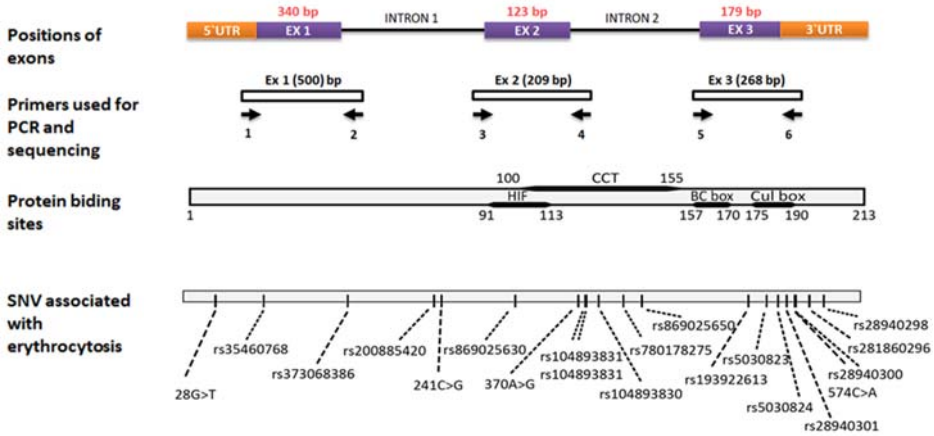

Figure 3: Presentation of VHL nucleotide and protein sequence structure with protein biding sites, collected variants associated with FE and designed PCR and sequencing primers. Legend: HIF – Interacting site with protein HIF; CCT – interacting site with chaperons Tric/CCT and Hsp 70; BC box – interacting sites with Elongin C and Elongin B; Cul box – interacting site with Cullin 2.

***EPAS1* sequence structure and variants.** Based on the current version of Ensembl browser transcript EPAS1-201 has 836 sequence variants. Literature review revealed positive association between FE and 12 sequence variants, which are located in exons 9, 12 and 16 [2, 4, 5]. Majority of variants (10 out of 12) have position in exon 12. Three variants in exon 12 (p.M535V, p.G537R, p.G537W) have a reference SNP ID number (rs137853036, rs137853036, rs137853037) and have been confirmed as a cause for ECYT4 in numerous studies. All variants in patients with FE discussed so far are heterozygous, suggesting that one mutant allele is sufficient enough to induce the phenotype. Majority of missense substitutions (exon 12) in connection with FE located C-terminal to the posttranslational hydroxylation site Pro-531, which highlight critical role of residues in this direction of the sequence for proteins stability and activity (Figure 4)[2].

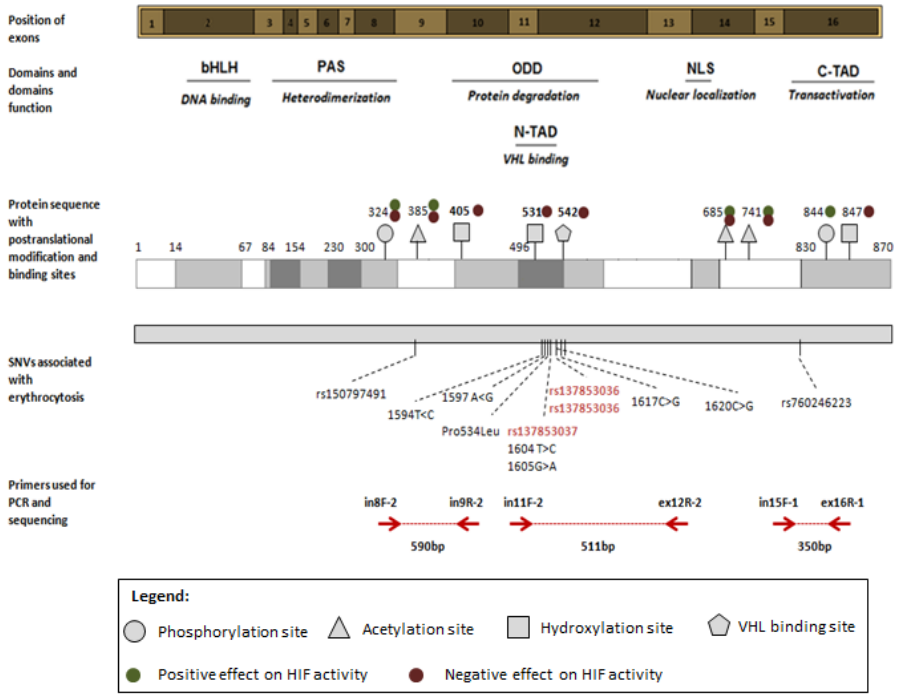

Figure 4: Presentation of EPAS1 nucleotide and protein sequence structure with domains and posttranslational-modification sites, collected variants associated with FE (variants that are confirmed as a cause for ECYT4 are indicated in red) and designed PCR and sequencing primers.

Mutational analysis of *EPO, EPOR*, *EPAS1* and *VHL* genes. Amplification and sequence analysis of target region of genes *EPO, EPOR, VHL and EPAS1* performed until now is shown in Table 1. None of the four patients have variants in coding regions. *EPO* variant was found in enhancer, but was not confirmed to be associated with FE[15]. Absence of sequence variants was also confirmed in two unaffected individuals. Further mutational analysis in other two exons of *EPAS1* gene and on additional patients for *EPO* and *EPOR* genes is currently in progress.

Table 1: Genotypes determined by mutation analysis

gene	region	P1	P2	P11	P21	C1	C3
EPOR	ex 8	wt	Wt	/	/	Wt	/
EPO	5' UTR	wt	Wt	/	/	Wt	/
	3' UTR	mut	Mut	/	/	Wt	/
VHL	ex 1	wt	Wt	Wt	wt	Wt	wt
	ex 2	wt	Wt	Wt	wt	Wt	wt
	ex 3	wt	Wt	Wt	wt	Wt	wt
EPAS1	ex 9	/	/	/	/	/	/
	ex 12	/	/	/	/	/	/
	ex 16	wt	Wt	Wt	wt	Wt	wt

Legend: /: not tested or undergoing testing, mut: sequence variants found in gene, wt: absence of sequence variants

Up to now, we analysed target regions of four genes involved in HIF pathway (*EPO, EPOR, VHL, EPAS1*) on four patients with *JAK2* negative erythrocytosis in Slovenia and none of them had variants associated with FE. However, our findings could serve as a part of diagnostic procedures in patients with FE in Slovenia. To elucidate the cause of idiopathic erythrocytosis, further screening for variants in HIF pathway or Hb is needed. As FE is rare disease, only few patients were screened. In aim to involve more patients, we are currently screen patients with *JAK2* negative erythrocytosis in Slovenia.

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IS *GYS1* GENE MUTATION CAUSATIVE FOR POLYSACCHARIDE STORAGE MYOPATHY (PSSM) IN SLOVENIAN COLD-BLOODED HORSE BREED?

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POVZETEK

Polisaharidna hrambna miopatija (angl. *polysaccharide storage myopathy*; PSSM) je motnja nalaganja glikogena, ki se pojavlja pri številnih pasmah konj. Sprva so jo odkrili pri quarter konjih, kasneje pa tudi pri nekaterih hladnokrvnih in drugih pasmah, kot so arabski konj, morgan in »*Tennessee Walking horse*«. Najpogostejši klinični znaki, povezani s PSSM pri quarter konjih vključujejo močne bolečine v mišicah, otrplost, šibkost, znojenje in odpor do gibanja. Nesinonimna mutacija povzroča nukleotidni polimorfizem (angl. *single nucleotide polymorphism*; SNP) (chr10:18940324 (G>A), EquCab2.0) znotraj zelo ohranjene regije gena *GYS1*, in povzroči zamenjavo aminokisline arginin (CGT) v histidin (CAT), kar je bilo pri številnih pasmah konj povezano s PSSM. Mutacija, ki povzroči pridobitev funkcije gena (angl. gain-of-function mutation) privede do skladiščenja abnormalne oblike glikogena v skeletnih mišicah in do poškodb mišic ob naporu. Pri slovenskem hladnokrvnem konju so opazili podobne simptome kot pri za PSSM obolelih belgijskih hladnokrvnih konjih. V pričujoči študiji smo z metodo PCR-RFLP določili genotip pri konju slovenske hladnokrvne pasme in s tem prvič identificirali prisotnost mutacije znotraj gena *GYS1* pri tej pasmi (v homozigotni obliki). Opisano motnjo je večinoma težko zaznati na fenotipski ravni, zato so bodo informacije o frekvenci mutacije znotraj gena *GYS1* pri tej pasmi zelo koristne za selekcijsko delo in oskrbo prizadetih konj, saj lahko začetne simptome preprečimo z ustreznim načinom prehrane in vadbe.

Ključne besede: *Equus caballus, GYS1*, polisaharidna hrambna miopatija, slovenski hladnokrvni konj

ABSTRACT

Polysaccharide storage myopathy (PSSM) is a glycogen storage disorder that occurs in several horse breeds. It was first discovered in quarter horses and later identified in draft horses and other breeds such as Arabians, Morgans, and Tennessee Walking horses. The most common clinical signs related to PSSM in quarter horses include firm painful muscles, stiffness, weakness, sweating and reluctance to move. A nonsynonymous mutation causing single nucleotide polymorphism (SNP) (chr10:18940324 (G>A), EquCab2.0) that changes arginine (CGT) to histidine (CAT) was identified within a highly conserved region of the skeletal muscle glycogen synthase gene *(GYS1)* and associated with PSSM in several horse breeds. The gain-of-function mutation results in abnormal glycogen accumulation in skeletal muscles and muscle damage with exertion. In Slovenian cold-blooded horses symptoms similar to those observed in the affected Belgian draft horses were

observed. In the present study, we genotyped a symptomatic Slovenian cold-blooded horse, using PCR-RFLP method, and identified the mutation in a homozygous form within the *GYS1* gene for the first time in this breed. The disorder is often difficult to observe at the phenotypic level, therefore, a more detailed information on the distribution of *GYS1* mutation in this breed is needed for selective breeding against the defect and appropriate management of the affected animals, as the onset of the symptoms can be prevented by the control of the diet and exercise.

Key words: *Equus caballus*, *GYS1,* polysaccharide storage myopathy, Slovenian cold-blooded horse

POSTERS – GENOMICS

Anamarija Habič
IDENTIFICATION OF FUNCTIONAL POLYMORPHISMS LOCATED WITHIN ULTRACONSERVED REGIONS (UCRs) IN HUMAN

Bojan Papić
COMPARISON OF ION PGM AND ILLUMINA HISEQ 2500 PLATFORMS BASED ON WHOLE-GENOME SEQUENCING OF *Listeria monocytogenes* ISOLATES

Tajda Mirič
IDENTIFICATION OF GENES ASSOCIATED WITH MILK FAT YIELD IN BROWN SWISS CATTLE POPULATION

Kristian Urh
IDENTIFICATION OF RISK FACTORS FOR CRYPTORCHIDISM AND INFERTILITY USING ARRAY COMPARATIVE GENOMIC HYBRIDISATION

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IDENTIFICATION OF FUNCTIONAL POLYMORPHISMS LOCATED WITHIN ULTRACONSERVED REGIONS (UCRs) IN HUMAN

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IZVLEČEK

Zelo ohranjene genomske regije (angl. *ultraconserved region*; UCR) so definirane kot zaporedja DNA, ki so stoodstotno ohranjena v ortolognih regijah genomov človeka, podgane in miši. Novejše raziskave kažejo, da so v UCR-jih prisotni tudi polimorfizmi. Večina UCR-jev še ni bila funkcionalno anotirana, zato je njihov vpliv na fenotip še precej neznan. Namen raziskave je bil: 1. na novo določiti genomske lokacije predhodno odkritih UCR-jev v skladu z najnovejšo različico genoma človeka, 2. določiti prekrivajoče gene, 3. identificirati polimorfizme znotraj UCR-jev in preveriti njihove morebitne povezave s fenotipom. Z uporabo orodja BioMart smo ugotovili, da se 75 % UCR-jev vsaj delno prekriva z geni. Znotraj ohranjenih regij smo določili 30139 polimorfizmov, od katerih jih ima 183 anotirano povezavo s fenotipom. Polimorfizmi znotraj UCR-jev so povezani z različnimi boleznimi, med drugim z družinsko adenomatozno polipozo, adolescentno idiopatsko skoliozo, amiotrofično lateralno sklerozo, mišičnimi distrofijami in spastično paraplegijo. Rezultati so osnova za nadaljnje odkrivanje funkcije UCR-jev in identifikacijo pomembnih regij znotraj teh odsekov.

Ključne besede: UCR, gen, polimorfizem, fenotip, bolezen

ABSTRACT

Ultraconserved regions (UCRs) have been defined as DNA sequences, which are absolutely conserved between orthologous regions of the human, rat and mouse genomes. Recent studies have proved the presence of polymorphisms within UCRs. Most of the UCRs have not yet been functionally annotated, therefore their effect on phenotype remains substantially unknown. The aims of the study were to: 1. remap previously reported UCRs according to the latest human genome release, 2. identify overlapping genes, 3. identify polymorphisms within UCRs and their potential associations with phenotype. Using BioMart data mining tool we showed that 75% of UCRs at least partially overlap gene regions. Our analysis performed by BioMart identified 30,139 polymorphisms within UCRs. Among these, 183 have been annotated to be associated with phenotype. They are associated with various diseases including familial adenomatous polyposis, adolescent idiopathic scoliosis, amyotrophic lateral sclerosis, muscle dystrophies, and spastic paraplegia. Our results serve as a basis for further investigation of UCRs' functions and identification of important regions within these segments.

Key words: UCR, gene, polymorphism, phenotype, disease

INTRODUCTION

Ultraconserved regions (UCRs) have been defined as genomic segments, which are fully conserved between orthologous regions of the human, rat and mouse genomes and are longer than 200 bps. Bejerano et al. identified 481 such sequences[1]. In most cases, exonic UCRs are located in genes involved in RNA processing, whereas nonexonic UCRs are located in introns or nearby genes encoding developmental and transcriptional regulators[1]. Stephen et al. greatly expanded the number of known UCRs as they reported 13,736 UCRs of at least 100 bps in length in the human genome, which are identical in at least three of five placental mammals: human, dog, cow, mouse, and rat [2].

UCRs can act as distal enhancers of nearby genes[1] or transcriptional coactivators[3], their role can also be associated with alternative splicing[4] and epigenetic modifications[5]. A large fraction of genomic UCRs were found to be transcriptionally active, encoding a particular set of ncRNAs called transcribed-ultraconserved regions or T-UCRs[6]. An increasing number of studies have proved that their expression is altered in multiple human cancers[6–9]. Recently, an association between hypoxia, a classic feature of the tumour microenvironment, and the modulation of T-UCRs has been reported[10].

Although UCRs have been defined as identical in multiple species' genomes, numerous polymorphisms within these regions in the human population have been identified[1, 11, 12]. As UCRs are under purifying selection, variants located within these regions may have a higher likelihood of association with phenotypic traits and diseases. Since the locations of UCRs are not yet included into genomic browsers, a vast majority of studies considering polymorphism-phenotype associations miss the fact that functional SNPs are located within UCRs. Therefore the role of these regions and the consequences of polymorphisms within them remain poorly understood. The aim of the present study was to remap previously reported UCRs according to the latest human genome release, identify genes and polymorphisms, overlapping UCRs and to analyse if those polymorphisms have been previously associated with phenotype or disease development.

MATERIAL AND METHODS

Nucleotide sequences of 13,736 UCRs ≥ 100 bps which are identical in at least three of five placental mammals were obtained from Stephen et al.[2]. 2,189 UCRs which are ≥ 200 bps in length were selected for further analysis. They were remapped according to the latest human genome release (GRCh38) using the Ensembl BLAT tool (BLAST-like Alignment Tool).

In order to identify genes overlapping UCRs we used BioMart data mining tool. Ensembl Genes 87 was set as database and *Homo sapiens* genes (GRCh38.p7) was chosen as dataset. UCRs' locations were then inserted into Filters/REGION/Multiple Chromosomal Regions. BioMart tool returned a table of genes and their genomic locations. A code in Python was used, which sorted the UCRs to the corresponding genes according to their genomic locations.

In the next step, we collected polymorphisms within 2,189 analyzed UCRs using BioMart. Ensembl Variation (release 86) was set as database and *Homo sapiens* Short Variants (SNPs and indels excluding flagged variants) (GRCh38.p7) were chosen as dataset. UCRs' genomic locations were inserted into Filters/REGION/Multiple Chromosomal Regions. A table of known polymorphisms and their locations was obtained. A code in Python was used in order to arrange polymorphisms to the UCRs within which they are located. Using adjusted settings in BioMart we also screened the UCRs for phenotype associated polymorphisms and retrieved scientific literature, reporting these associations. Scientific articles obtained with BioMart were manually reviewed in order to verify given associations. Additional scientific articles reporting the associations were found manually in the databases listed in the results table.

RESULTS AND DISCUSSION

UCRs' genomic distribution and statistics. The analysis of the genomic distribution of selected UCRs revealed that they are located within all human chromosomes, except on chromosome Y (Fig. 1). The ratio between the number of UCRs and chromosome length is substantially lower in the case of chromosome 21 when compared to other chromosomes. Chromosome 2 contains the highest number of UCRs per chromosome length.

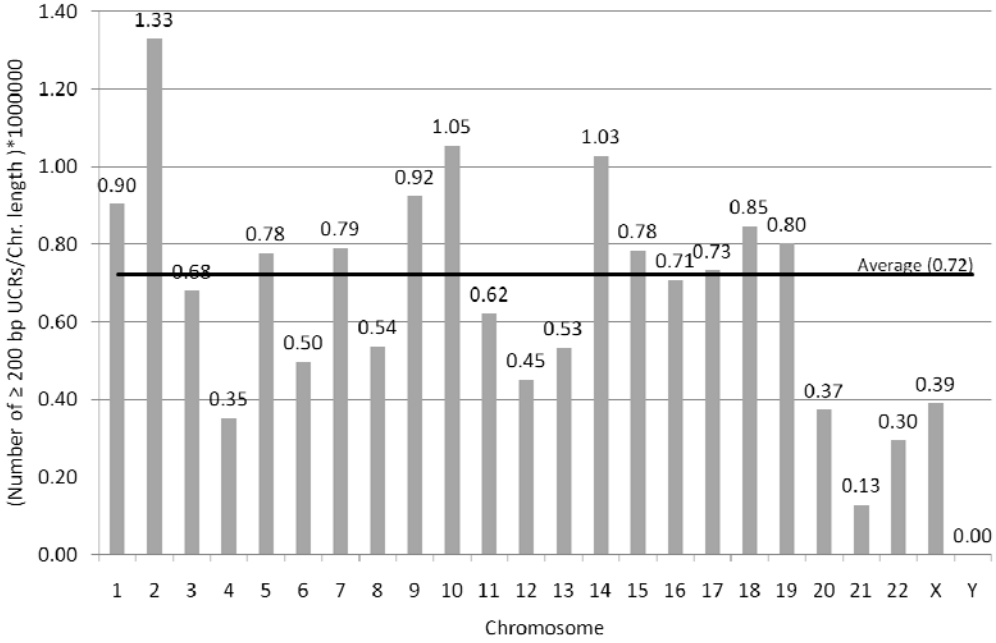

Figure 1: Ratio between number of ≥ 200 bps long UCRs and chromosome length.

The average length of 2,189 analyzed UCRs is 287 bps. 46.7% of UCRs are 200–249 bps in length and 98.4% are shorter than 600 bps (Fig. 2). Both longest UCRs (UCR 13336, 1,087 bps and UCR 13347, 1,085 bps, respectively) are located within the *POLA1* (DNA polymerase alpha 1, catalytic subunit) gene on chromosome X. POLA1 plays a crucial role in the initiation of DNA replication and high conservation is expected due to the protein's important function which requires a specific protein structure. However, both longest UCRs are located within introns at the 3' end of the gene. As suggested by Bejerano et al., who also identified UCRs in this region, it is possible that their function is unrelated to *POLA1*, but that they instead form a cluster of enhancers of *ARX*, which is located near the end of the *POLA1*[1]. ARX is a transcription factor required for normal brain development. It is associated with a host of X-linked diseases including epilepsy, mental retardation and cerebral malformations[13].

UCR 2360 consists of 882 bps and is located on chromosome 11; it overlaps two genes: *ELP4* (Elongator acetyltransferase complex subunit 4) and *Z83001.1* (antisense to *ELP4*). It is located within an intron at the 3' end of the *ELP4* gene. In the proximity of UCR 2360 but on the reverse strand PAX6 is located, which is one of the key developmental regulatory genes with pleiotropic functions. Transcriptional control of *PAX6* expression depends on a large array of cis-elements. A mutation within an UCR (uc.325) in intron 9 of the *ELP4* (rs606231388) was confirmed to be causing a panocular disease aniridia. The mutation disrupts an autoregulatory PAX6 binding site, causing loss of enhancer activity, resulting in defective maintenance of *PAX6* expression[14]. It is possible that UCR 2360 hosts some important regulatory region as well.

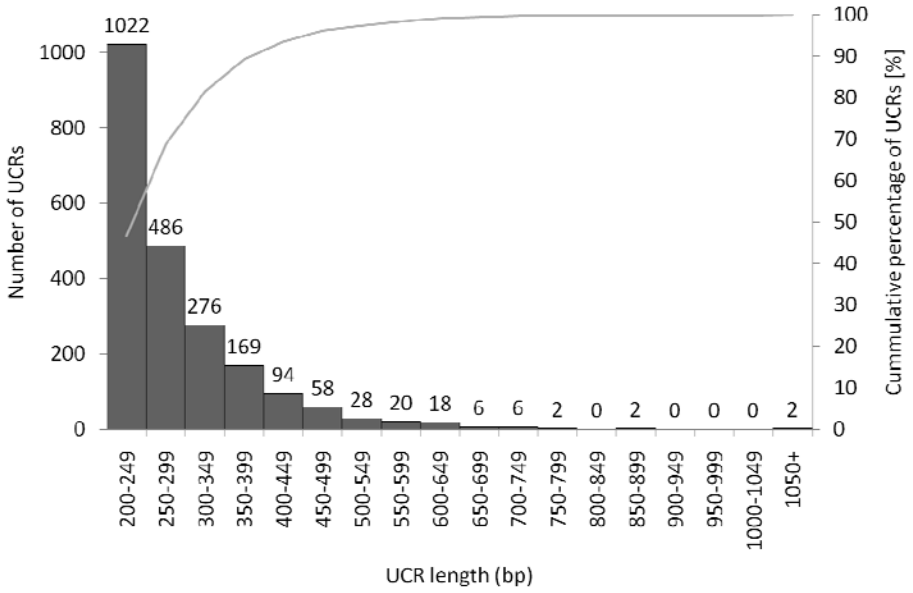

Figure 2: Length distribution of analyzed UCRs

Genes overlapping UCRs. Among 2,189 UCRs 24.7% are intergenic, while 75.3% are located within 874 genes of several different types, most frequently protein-coding genes and genes encoding lncRNAs (Table 1). It has been discussed previously that a UCR located within a certain gene is not necessarily related to this gene; it can for example act as an enhancer of another nearby gene. In 337 among 874 genes more than one UCR is present. Interestingly, 24 genes contain more than nine UCRs. *FOXP2* (forkhead box P2) gene contains the highest number, namely 28 UCRs.

Table 1: Number of genes of certain biotype, which contain UCRs

Biotype	Number of genes
Protein coding	627
lincRNA	119
Antisense	90
Processed transcript	16
Other lncRNA: sense overlapping; sense intronic; 3' overlapping ncRNA; noncoding; bidirectional lncRNA	10
Pseudogenes: polimorfic pseudogene; transcribed pseudogene	5
ncRNA: misc RNA; rRNA	4
To be experimentally confirmed	3

Polymorphisms within UCRs. Within the 2,189 screened UCRs 30,139 polymorphisms were found using BioMart. However, some of them have identical genomic locations, since different names were found in different source bases (dbSNP, HGMD-PUBLIC, ESP, PhenCode, ClinVar). Each of analyzed 2,189 UCRs contains at least one polymorphism. Within 215 UCRs five or less polymorphisms are present; seven UCRs contain only one polymorphism. The highest number of polymorphisms (70) is located within UCR 5114, which is 554 bps in length and is located on chromosome 16 within the *NFAT5* gene (nuclear factor of activated T-cells 5). However, UCRs containing high numbers of polymorphisms are rare; only 22 UCRs contain more than 50

polymorphisms. Nevertheless, these regions would be worth paying attention, since it is hard to explain their extreme interspecies conservation.

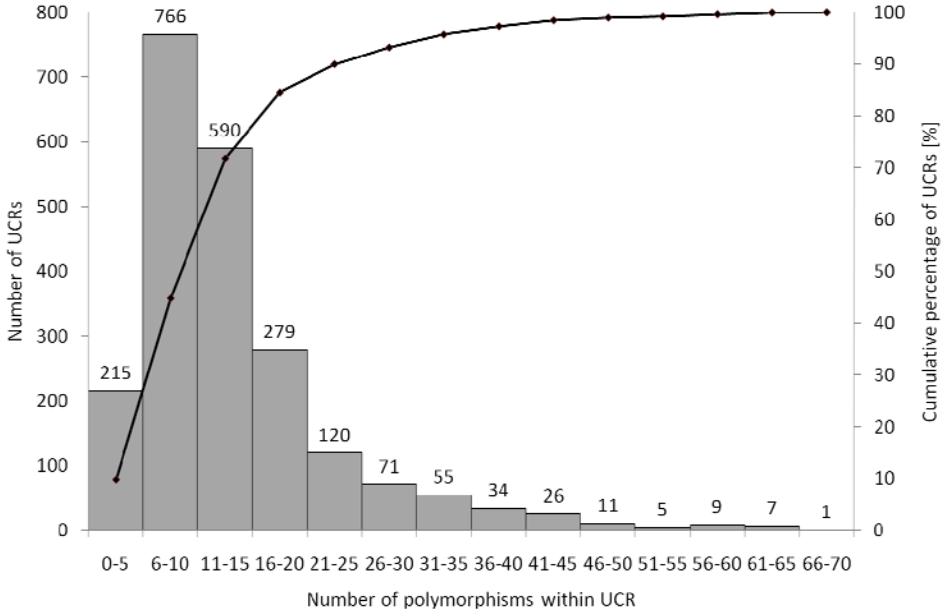

Figure 3: Graphical display of number of UCRs according to number of polymorphisms within them

Phenotype associated polymorphisms within UCRs. Among 30,139 polymorphisms within UCRs, 183 have been annotated to be associated with diseases or phenotypic traits in the Ensembl genome browser. Associated phenotype description was given for 76 among 183 polymorphisms with phenotype annotation, while for the rest of the polymorphisms it was not specified (annotations from ClinVar database) or it was not publicly available (annotations from HGMD database). For 37 among 183 polymorphisms it was possible to obtain published literature reporting associations with phenotype. Most of the reviewed scientific papers do not mention the fact that the location of the studied polymorphism is within an UCR.

UCR polymorphisms are associated with various diseases, including familial adenomatous polyposis, adolescent idiopathic scoliosis, amyotrophic lateral sclerosis, muscle dystrophies, spastic paraplegia and many others, according to the annotations in the Ensembl genome browser. A vast majority of phenotype associated polymorphisms is located within protein coding genes. Only four polymorphisms with annotated phenotypes are intergenic. One of these, however, is rs11190870 SNP, showing the strongest association with adolescent idiopathic scoliosis in several genome wide association studies[15].

All of the 183 phenotype associated polymorphisms which have been found, are located within only 48 UCRs (among 2,189 screened UCRs), meaning that in some UCRs, i.e. 16 UCRs, multiple phenotype associated polymorphisms are present. Six UCRs contain at least ten phenotype associated polymorphisms. For example, UCR 10358 on chromosome 5 within the *APC* gene (adenomatous polyposis coli protein) includes the highest number, namely 35 such polymorphisms. They are all associated with familial adenomatous polyposis or other similar conditions. On chromosome X, UCR 13372 within *DMD* (codes for dystrophin) and UCR 13568 within *PLP1* (codes for myelin proteolipid protein) contain 21 and 20 phenotype associated polymorphisms, respectively. The uneven distribution of phenotype associated polymorphisms across UCRs revealed in our study suggests that at least some of the UCRs affect phenotype. As such, they could be potential targets of

new therapeutic approaches. Results presented here contribute to a better understanding of the UCRs' roles, although much further study will be needed.

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COMPARISON OF ION PGM AND ILLUMINA HISEQ 2500 PLATFORMS BASED ON WHOLE-GENOME SEQUENCING OF *LISTERIA MONOCYTOGENES* ISOLATES

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POVZETEK

Ugotavljanje polimorfizmov posameznih nukleotidov (ang. *single nucleotide polymorphism*, SNP) je sestavni del analize bakterijskih in drugih genomov. Čeprav veljata tehnologiji sekvenciranja naslednje generacije (ang. *next-generation sequencing*, NGS) Illumina in Ion Torrent za zanesljivi in ponovljivi, so lahko ugotovljeni polimorfizmi tudi lažni – posledica napak med pripravo knjižnic ali sekvenciranjem, premajhne pokritosti genomov in/ali neprimerne bioinformacijske obdelave. V okviru našega dela smo na primeru treh izolatov *Listeria monocytogenes* ugotavljali primerljivost platform Ion PGM (400 bp) in Illumina HiSeq2500 (2×100 bp). V zaporedjih genomov, ki smo jih pridobili z različnima platformama, smo polimorfizme posameznih nukleotidov ugotavljali z dvema pristopoma: (*i*) na osnovi poravnave sestavljenih zaporedij (nezaključenih genomov) in (*ii*) na osnovi poravnave odčitkov na referenčni genom (ugotavljanje polimorfizmov). Kljub več kot 40× pokritosti genomov in uporabi ustreznih bioinformacijskih filtrov za nadzor kakovosti smo na osnovi poravnave nezaključenih genomov z algoritmom progressiveMauve ugotovili 26–48 polimorfizmov. Po drugi strani smo na osnovi poravnave odčitkov na bližje soroden referenčni genom s spletnim orodjem CSI Phylogeny ugotovili 2–3 zanesljive polimorfizme. Z upoštevanjem minimalne razdalje med polimorfizmi 10 bp je se njihovo število dodatno zmanjšalo na 0–3. Naša raziskava kaže na dobro primerljivost tehnologij in nakazuje, da sta uporaba ustreznega pristopa in bioinformacijskih filtrov za nadzor kakovosti ključna za zanesljivo ugotavljanje genetskih razlik med ozko sorodnimi izolati. Večje število ugotovljenih polimorfizmov na osnovi sestavljenih zaporedij je verjetno posledica izgube informacij o pokritosti in zastopanosti variant.

Ključne besede: SNP, NGS, Illumina, Ion Torrent

ABSTRACT

Single nucleotide polymorphism (SNP) calling methods are an integral part of comparative genomics. Although the two widely used next-generation sequencing technologies – Ion Torrent and Illumina – are considered to be reliable and reproducible, false positive hits may arise due to errors during library preparation or sequencing, insufficient genomic coverage, and/or inadequate bioinformatic analyses. In the present work, three *Listeria monocytogenes* isolates were sequenced using Ion PGM (400 bp) and Illumina HiSeq2500 (2×100 bp). Two approaches for SNP calling were employed to assess the performance of both methods: (*i*) based on the alignment of draft genomes and (*ii*) based on reads mapped to the reference genome. Although 40× coverage was obtained and appropriate bioinformatic quality control filters were used, SNP calling based on the alignment of draft genomes with progressiveMauve algorithm identified 26–48 SNPs. On the other hand, 2–3 high-

quality polymorphisms were identified with SNP calling based on read mapping to a closely related reference genome with CSI Phylogeny tool. This number further decreased to 0–3 when applying the minimum distance between SNPs of 10 bp. Our results show good comparability of technologies and suggests that the use of an appropriate SNP-calling approach and bioinformatic quality control filters is crucial for the reliable identification of genetic differences between closely related isolates. A larger number of identified SNPs from the aligned draft genomes is probably caused by the loss of information on the coverage and frequency of the variant.

Key words: SNP, NGS, Illumina, Ion Torrent

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IDENTIFICATION OF GENES ASSOCIATED WITH MILK FAT YIELD IN BROWN SWISS CATTLE POPULATION

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IZVLEČEK

S proizvodnimi lastnostmi mleka so povezani številni geni in kromosomske regije. Lokusi kvantitativnih lastnosti (QTL) za količino maščobe v mleku se pri govedu nahajajo na vseh kromosomih. Vendar so si objavljeni podatki nasprotujoči in kandidatni geni za številne QTL-e še niso znani. Namen te raziskave je bil določiti gene za količino maščobe v mleku v populaciji rjavega goveda (BSW). Analiza je bila izvedena na podatkih zadnjega mednarodnega obračuna plemenskih vrednosti mlečnih pasem goveda Interbull-centra. Študijska skupina je vključevala 26.203 genotipiziranih živali, od katerih je 6.542 živali imelo tudi mednarodno oceno plemenske vrednosti, preračunano na slovensko merilo. Genotipizacija je bila izvedena z uporabo različnih tipov DNA-mikromrež, med njimi BovineSNP50 BeadChip. Analizirali smo genomsko razporeditev 20 polimorfizmov, najbolj značilno povezanih s količino maščobe v mleku. Rezultati so razkrili štiri genomske regije z večjo gostoto polimorfizmov na kromosomih 5, 6, 9 in 25. Šest od 20 analiziranih polimorfizmov se nahaja znotraj petih genov: *SLC25A43*, *NAV3*, *BFAR*, *HS3ST6* in *TMPRSS11F*. Gen *NAV3* je posebej zanimiv, saj vključuje dva polimorfizma, povezana s količino maščobe v mleku. Nekateri signali so se prekrivali s predhodno poročanimi QTL-i za podobne lastnosti pri drugih populacijah goveda. Za določanje zanesljivejših biooznačevalcev bi bilo potrebno analizo opraviti tudi pri drugih pasmah goveda.

Ključne besede: govedo, DNA-mikromreža, količina maščob v mleku

ABSTRACT

Several genes and chromosomal regions have been associated with milk traits. Reported QTLs associated with milk fat yield are distributed on all cattle chromosomes. However, published data are opposing and several QTL candidate genes are unknown. The aim of the present study was to identify genes, associated with milk fat yield in Brown Swiss (BSW) cattle population. The analysis was performed using the data available from the international genetic evaluation of dairy cattle at Interbull Centre. Study group included 26,203 genotyped animals including 6,542 with international breeding values on Slovenian scale. Genotyping was performed with various types of DNA microarrays, including BovineSNP50 BeadChip. We analyzed genomic distribution of 20 polymorphisms, most significantly associated with milk fat yield. The results revealed four genomic regions with higher density of polymorphisms on chromosomes 5, 6, 9, and 25. Six out of 20 polymorphisms are located within five genes: *SLC25A43*, *NAV3*, *BFAR*, *HS3ST6*, and *TMPRSS11F*. Gene *NAV3* is of special importance, since it includes two polymorphisms associated with milk fat yield. Some signals overlapped previously reported QTLs for similar traits in other cattle populations. For identification of more robust markers, the analysis should also be performed in other cattle breeds.

Key words: cattle, DNA microarray, milk fat yield

INTRODUCTION

Genome-wide association study (GWAS) is a powerful approach for identification of genes and chromosomal regions associated with dairy phenotypes and is useful for gene-based and genome selection (Harder et al., 2006). Several genes and chromosomal regions have been associated with a large number of milk traits. Reported QTLs associated with milk fat are distributed on all cattle chromosomes and are systematically catalogued in the QTLdb (Hu et al., 2016). For example, in Holstein cattle a QTL for milk fat yield has been identified in BTA13 (Cole et al., 2011). Additionally, *PPARGC1A* gene has been suggested to be involved in genetic variation underlying the QTL for milk fat synthesis on BTA6 (Weikard et al. 2005). The strongest signal for milk production traits, including fat yield in Brown Swiss cattle was identified on chromosome 25 (Guo et al., 2012). This study also included samples from Slovenian Brown Swiss cattle population. Published data are opposing and several QTL candidate genes are still unknown. The aim of the present study was to identify genes, associated with milk fat yield in Brown Swiss cattle population.

MATERIAL AND METHODS

Databases and bioinformatics tools. Ensembl release 90 was used to retrieve genomic locations of polymorphisms and overlapping protein-coding genes (http://www.ensembl.org/index.html). Conversion of commercial BovineSNP50 BeadChip polymorphism names to reference SNP (rs) ID was performed using SNPchiMp tool (http://bioinformatics.tecnoparco.org/SNPchimp/) (Nicolazzi et al., 2014). Quantitative trait loci data were downloaded from QTLdb, release 33 (https://www.animalgenome.org/cgi-bin/QTLdb/index) (Hu et al., 2016). Ensembl genes which have not yet been manually annotated were identified using information of orthologous genes from Ensembl genomic browser and NCBI database (https://www.ncbi.nlm.nih.gov/).

Samples, genotyping and statistical analysis. The analysis was performed using the data available from the international genetic evaluation of dairy cattle at Interbull Centre (Jorjani et al., 2011). Study group included 26,203 genotyped animals including 6,542 with international breeding values on Slovenian scale (analysis performed in March, 2017). Genotyping was performed using various types of DNA microarrays, including BovineSNP50 BeadChip, Geneseek GPP, IDV v.2 and v.3) and all results were unified according to the Interbull SNP index. Statistical analysis was performed as described previously (Guo et al., 2012). Estimated regression coefficients from SNP equation for milk fat yield on Slovenian scale were used. Each regression coefficient represent additive genetic effect of each SNP on milk fat yield.

RESULTS AND DISCUSSION

Results of the genome-wide association analysis. In the present study we performed DNA-microarray analysis for identification of genes associated with milk fat yield in Brown Swiss cattle breed. The results revealed that the first 280 polymorphisms, most significantly associated with milk fat yield contributed to phenotypic variability in 3% and the first 100 most significantly associated polymorphisms contributed 1.22% to the researched trait. In the present study we analyzed genomic

distribution of 20 polymorphisms most significantly associated with milk fat yield, which explained 0.29% of the phenotypic variability.

Genomic distribution of polymorphisms associated with milk fat yield. The highest number of SNPs associated with milk fat yield is located on the chromosomes: 5 (four SNPs), 6 (four SNPs), 25 (three SNPs), 9 (two SNPs), and 20 (two SNPs). One polymorphism per chromosome is located on chromosomes 13, 14, 17, 28, and X. Genomic location of twenty SNPs significantly associated with milk fat yield is presented in Table 1. The table also includes information of the nearest protein-coding genes, their orientation (sense, antisense) and the location of polymorphisms relative to the gene (upstream, downstream or within the gene).

Six out of these 20 polymorphisms are located in five genes: rs42197808 (in the *SLC25A43* gene), rs42774123 and rs43710106 (both in the *NAV3* gene), rs110426340 (in the *BFAR* gene), rs109557202 (in the *HS3ST6* gene), and rs41655005 (in the *TMPRSS11F* gene). These six polymorphisms are intronic.

Fourteen out of 20 polymorphisms are intergenic and located in the vicinity of 24 protein-coding genes: *SLC4A4*, *GC*, *ENSBTAG00000046399*, *CPPED1, RBM19, TBX5, DCK*, *SOCS2, ENSBTAG00000005107*, *SRGN*, *ENSBTAG00000014558, ENSBTAG0000013213, STK3, SLC1A3, RANBP3L*, *KCNJ8, LDBH, KIAA0408, ECHDC1, MAFB, TOP1, NPFFR2*, *SLIT3* and *ENSBTAG00000047333*. For five of these protein-coding genes the official gene name is not yet available in the Ensembl database. They are named with the Ensembl ID (ENSBTA#). Names for four out of these five genes were obtained using the information of their orthologous genes: *ENSBTAG00000046399* (*SHISA9*), *ENSBTAG00000014558* (*DDX21*), *ENSBTAG00000013213* (OSR2), and *ENSBTAG00000005107* (*CRADD*). For the Ensembl gene *ENSBTAG00000047333* 34 orthologous genes are known, but all of them have only Ensembl names, so it was not possible to determine annotated gene name.

The results revealed that polymorphisms are not uniformly distributed throughout the genome. There are four genomic regions with higher density of polymorphisms associated with milk fat yield; on chromosomes 5, 6, 9 and 25. Four SNPs are located on the chromosome 5, two of them are located within the gene *NAV3* (rs42774123 and rs43710106). On the chromosome 6 four polymorphisms are located. One polymorphism is located within the *TMPRSS11F* gene. Three polymorphisms (rs110527224, rs110354582, and rs42766480) are located in vicinity of four genes *DCK*, *SLC4A4*, *GC*, and *NPFFR2* gene. These three polymorphisms are located at the distance of 0.78 Mbp (Figure 1).

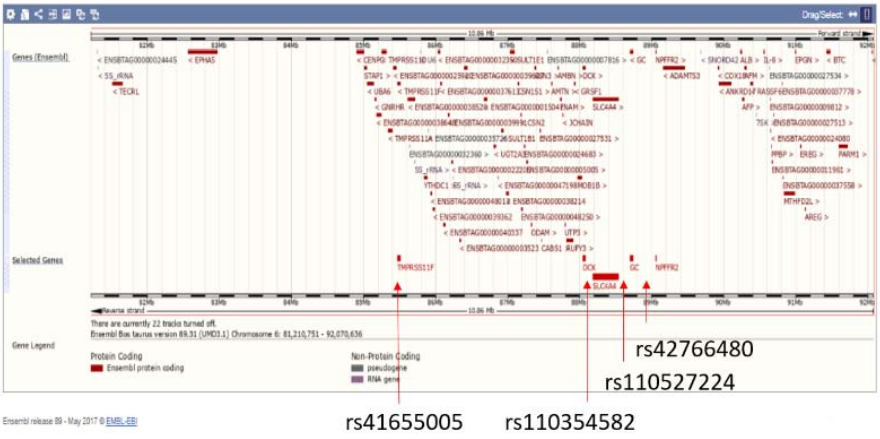


Figure *4*: Presentation of the chromosome BTA6 with four polymorphisms associated with milk fat yield in Brown Swiss cattle breed. One polymorphism is located within the *TMPRSS11F* gene and three polymorphisms are located in vicinity of the *DCK, SLC4A4*, *GC*, and *NPFFR2* genes.

Table *3*: Genomic location of twenty SNPs significantly associated with milk fat yield and associated genes.

rs SNP ID	Chromosome	Nearest gene 1			Nearest gene 2		
		Gene name	Orientation (sense/ antisense)	SNP location	Gene name	Orientation (sense/ antisense)	SNP location
rs110527224	6	*SLC4A4*	Sense	Downst.	*GC*	Antisense	Downst.
rs42055812	25	*ENSBTAG00000046399 (SHISA9)*	Sense	Downst.	*CPPED1*	Antisense	Upst.
rs41625122	17	*TBX5*	Sense	Downst.	*RBM19*	Sense	Upst.
rs42197808	X	*SLC25A43*	Sense	In	/	/	/
rs42774123	5	*NAV3*	Sense	In	/	/	/
rs110354582	6	*DCK*	Sense	Downst.	*SLC4A4*	Sense	Upst.
rs41654779	5	*SOCS2*	Sense	Downst.	*ENSBTAG00000005107 (CRADD)*	Sense	Upst.
rs110103383	28	*ENSBTAG00000014558 (DDX21)*	Sense	Downst.	*SRGN*	Sense	Upst.
rs110405331	14	*ENSBTAG00000013213 (OSR2)*	Antisense	Upst.	*STK3*	Sense	Upst.
rs41943459	20	*SLC1A3*	Antisense	Upst.	*RANBP3L*	Sense	Upst.
rs109014036	9	*KIAA0408*	Sense	Downst.	*ECHDC1*	Sense	Upst.
rs109628799	5	*KCNJ8*	Sense	Downst.	*LDHB*	Sense	Upst.
rs41579880	9	*KIAA0408*	Sense	Downst.	*ECHDC1*	Sense	Upst.
rs110426340	25	*BFAR*	Sense	In	/	/	/
rs109557202	25	*HS3ST6*	Antisense	In	/	/	/
rs110573257	13	*MAFB*	Antisense	Downst.	*TOP1*	Sense	Upstr.
rs41655005	6	*TMPRSS11F*	Antisense	In	/		/
rs43710106	5	*NAV3*	Sense	In	/		/
rs42766480	6	*GC*	Antisense	Upst.	*NPFFR2*	Sense	Upst.
rs109346620	20	*ENSBTAG00000047333*	Sense	Downst.	*SLIT3*	Antisense	Downst.

Downst. – downstream; Upst. – upstream; In – in the gene

Two polymorphisms located in the chromosome 9 (rs109014036 and rs41579880) are located in vicinity of two genes; *KIAA0408* and *ECHDCI* (Figure 2). The distance between these two polymorphisms is 27,879 bps. Three polymorphisms are located on the chromosome 25, including two at the distance of 1.88 Mbp (rs42055812 and rs110426340).

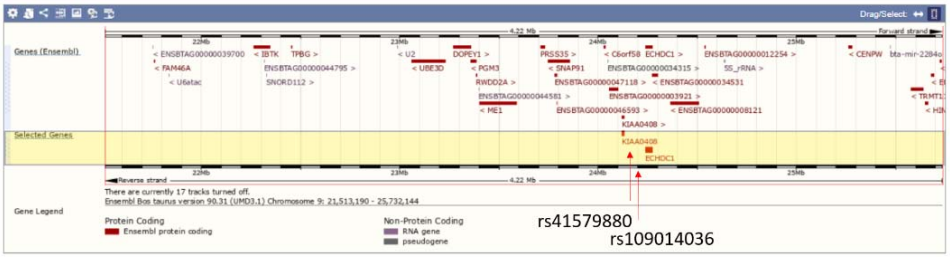
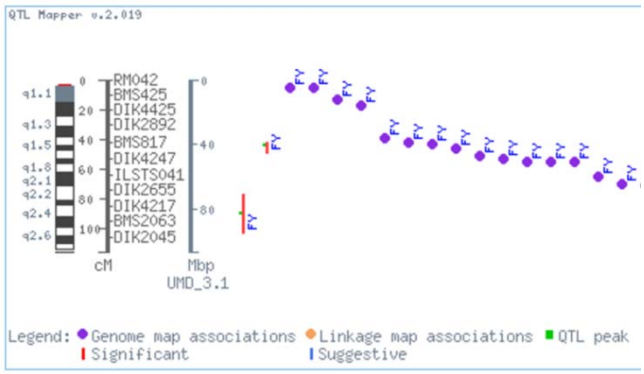


Figure 2: Presentation of the chromosome BTA9 with two polymorphisms associated with milk fat yield in Brown Swiss cattle breed. Polymorphisms are located between *KIAA0408* and *ECHC1* genes.

Comparison of genomic distribution between polymorphisms and QTL. We also performed comparison of the genomic location of 20 researched polymorphisms with location of previously identified QTLs associated with milk fat yield. The analysis revealed genomic overlaps. For example, on the chromosome BTA9 two polymorphisms (rs41579880 and rs109014036) overlap the region of two previously identified QTLs (Figure 3). QTL#1687 spans the region 18.2–26.7 Mbp and it has been identified using genome-wide scan of 221 microsatellite loci in Holstein breed (Schnabel et al., 2005). QTL #121799 spans the region 24.1–24.2 Mbp and it has been identified in a genome wide CNV analysis in Holstein breed (Xu et al., 2014). Candidate genes associated with this QTL are not yet known. These two QTL regions span large genomic regions. Additional studies are needed to identify the associated loci.

A.



B.

QTL Map Information		
	Chromosome:	9
	QTL Peak Location:	n/a
	QTL Span:	n/a 24.1-24.2 (Mbp)
Flanking markers	Upper, "Suggestive":	n/a
	Upper, "Significant":	rs42490238
	Peak:	
	Lower, "Significant":	rs109014036
	Lower, "Suggestive":	n/a
	Analysis type:	Association

Figure 3: Presentation of QTLs, associated with milk fat yield (FY) in chromosome BTA9. Two polymorphisms (rs41579880 and rs109014036) are located within this region and they overlap with two QTL. QTL#1687 spans the region 18.2–26.7 Mbp (A). QTL #121799 spans the region 24.1–24.2 Mbp (B).

Conclusion. Six out of 20 analyzed polymorphisms are located within five genes (*SLC25A43, NAV3, BFAR, HS3ST6*, and *TMPRSS11F*). Gene *HS3ST6* has previously been reported to be associated with milk fat yield (Guo et al., 2012). To our knowledge, the other four genes have not yet been associated with milk fat yield in cattle. In this study we therefore confirmed one and identified four novel candidate genes for this trait. Gene *NAV3* is of special importance since it includes two polymorphisms associated with milk fat yield. In the present study we analyzed genomic location of 20 polymorphisms most significantly associated with milk fat yield. The study should be extended to other polymorphisms, for example to 280 polymorphisms explaining 3% of the phenotypic variability. For identification of more robust markers, the analysis should also be performed on other cattle breeds.

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IDENTIFICATION OF RISK FACTORS FOR CRYPTORCHIDISM AND INFERTILITY USING ARRAY COMPARATIVE GENOMIC HYBRIDISATION

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IZVLEČEK

Kriptorhizem je pogosta urogenitalna anomalija, ki se pojavlja pri 2–4 % polno donošenih rojstev in predstavlja tveganje za razvoj neplodnosti. Je večfaktorska bolezen, k razvoju katere prispevajo tudi genetski vzroki. Neplodnost se pojavlja pri ~7 % moških; genetski vzroki obsegajo 15–30 % moške neplodnosti. Izvedba molekularnih analiz na ravni celotnega genoma omogoča določanje dejavnikov tveganja za razvoj kriptorhizma in neplodnosti. V raziskavi smo analizirali različice števila kopij (CNV) pri 28 bolnikih s kriptorhizmom in/ali neplodnostjo z uporabo metode primerjalne genomske hibridizacije na mikromrežah (aCGH). Določili smo 23 CNV-jev, ki predstavljajo kandidatne dejavnike tveganja za razvoj kriptorhizma in/ali neplodnosti. Območja CNV zajemajo 18 genov in več regulatornih elementov. V prihodnosti bo potrebno raziskati ali gre za različice *de novo*, preveriti klinični vpliv identificiranih CNV-jev in CNV-je potrditi z uporabo dodatnih metod.

Ključne besede: kriptorhizem, neplodnost, različice števila kopij

ABSTRACT

Cryptorchidism is a frequent urogenital anomaly, it presents 2–4% of full term male births, and is associated with risk of infertility development. It is a multifactorial disease, which is at least in part determined by genetic causes. Infertility appears in ~7% of males; genetic causes are associated with 15–30% of male infertility. Molecular analyses performed at the genome-wide level allow identification of risk factors for development of cryptorchidism and infertility. In the present study we performed analysis of copy number variations (CNV) in 28 patients with cryptorchidism and/or infertility using array comparative genomic hybridisation (aCGH) method. We identified 23 CNVs that present candidate risk factors for development of cryptorchidism and/or infertility. Regions within CNVs include 18 genes and several regulatory elements. In the future studies it will be necessary to check if these are *de novo* variants, analyse their clinical significance and validate identified CNVs with additional methods.

Key words: cryptorchidism, infertility, copy number variation

INTRODUCTION

Cryptorchidism is a urogenital anomaly, in which the testis does not descend through the inguinal canal into the scrotum. It is present in 2–4% of full term male births[1]. Cryptorchidism is associated with a higher probability of infertility and tumours. Cryptorchidism is at least in part

determined by genetic causes. A database of cryptorchidism associated loci was first developed in 2014 and updated in 2016[2, 3]. Infertility appears in ~7% of all males and genetic causes are associated with 15%–30% of male infertility[4, 5]. Several genetic causes, such as single nucleotide polymorphisms (SNPs), are associated with the development of male infertility and are present on autosomes, the X chromosome and in mitochondrial DNA (mtDNA). Genetic malformations can also result in hormonal imbalances[6]. In cases where the cause of infertility is unknown, it is classified as idiopathic[7].

Array comparative genomic hybridisation (aCGH) method enables identification of copy number variations (CNVs) on a genome-wide level. The method is based on measuring of differences in fluorescence of test and reference DNA that have comparatively hybridised to probes for different locations in the genome on the microarray. The analysis software identifies information on copy number variation of the test genome against the reference genome. The main advantage of the aCGH method against other molecular genetic methods is the ability to identify aneuploidies, deletions, duplications and/or amplifications of loci present on the microarray in one test. Results obtained with the aCGH are commonly validated with fluorescent *in situ* hybridisation (FISH), or quantitative PCR[8].

Knowledge of the CNV effects in patients with male infertility and cryptorchidism is still limited. The aim of the present study was therefore to analyse, if CNVs present risk factors for development of cryptorchidism and/or infertility in a group of patients with cryptorchidism and/or idiopathic infertility using aCGH method.

MATERIAL AND METHODS

Patients. The study group included 28 male participants with cryptorchidism and/or idiopathic infertility. Fourteen participants had cryptorchidism in the clinical picture, 13 had idiopathic infertility and one participant had cryptorchidism and idiopathic infertility. The idiopathic infertility group included 11 participants with azoospermia and two participants with cryptozoospermia. One participant with cryptozoospermia also had cryptorchidism present in the clinical picture. Clinical characteristics of patients were described previously[9]. Orchidopexy was performed on all participants with cryptorchidism. The study was approved by the Medical ethics committee of Republic of Slovenia (reference number: 73/05/12). A written consent has been obtained from all participants included in the study.

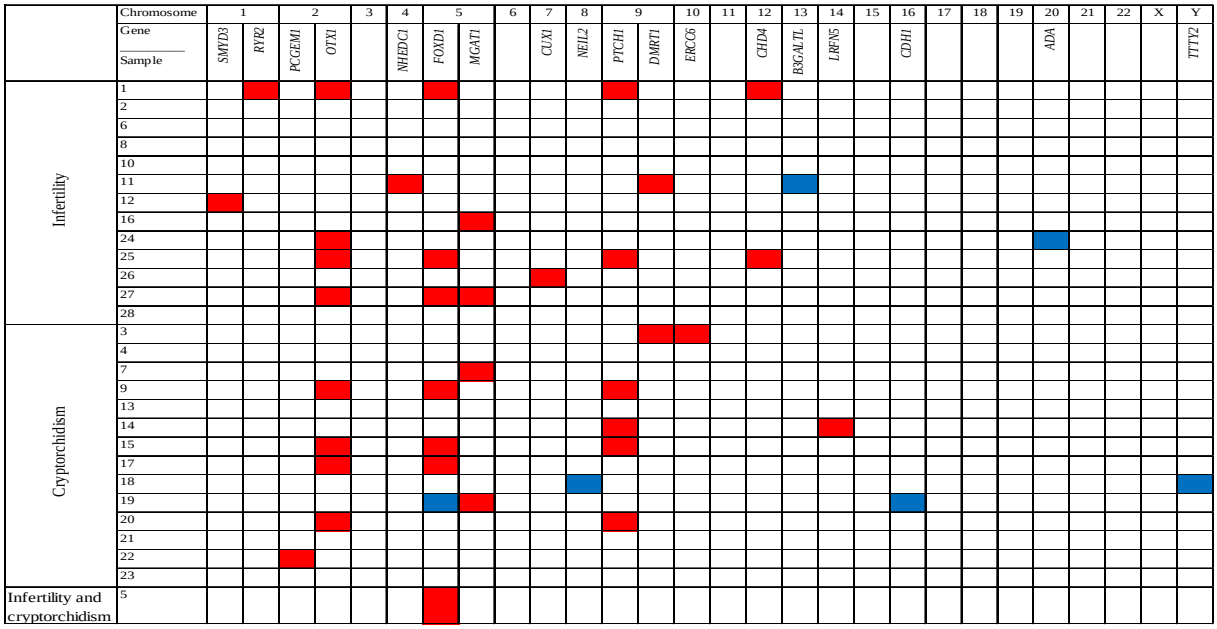
Microarrays. Microarrrays SurePrint G3 Unrestricted CGH (4x180K) from the manufacturer Agilent (USA) were used in the experiment. The microarrays include 16,605 (88. 8 %) genes included in the RefSeq database. The average distance between genomic probes on the microarray is 11 kbp.

Array comparative genomic hybridisation (aCGH). The experiment was performed as reported previously[10]. The protocol was modified to comply with the manufacturers current protocols for the Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis[11]. Identified CNVs were further compared with the data deposited in the UCSC genomic browser (https://genome.ucsc.edu/), DECIPHER (https://decipher.sanger.ac.uk/), ISCA (http://dbsearch.clinicalgenome.org/search/), OMIM (https://www.omim.org), and PubMed databases (https://www.ncbi.nlm.nih.gov/pubmed/). The UCSC genome browser was used for identification of exons, introns, and regulatory elements in the CNVs. Data from the ClinGen database (information on pathogenic CNVs) (https://www.clinicalgenome.org/) and Database of Genomic Variants (DGV) (information on CNVs present in the general population) (http://dgv.tcag.ca/dgv/app/home?ref=GRCh37/hg19) was also obtained. The DECIPHER database was used for identification of patients included in the database that had cryptorchidism/infertility

and similar sized gains/losses as the analysed participants. The database also reports clinical significance and mode of inheritance The ISCA database was used for identification of CNVs with known clinical significance that were included in the database. The OMIM database and PubMed were used for identification of association between the CNVs and cryptorchidism and/or infertility.

RESULTS AND DISCUSSION

In the aCGH experiment we identified 23 CNVs in 28 participants. Out of 23 CNVs, 18 contain genes and five CNVs are intergenic. Figure 1 is a graphical representation of the 18 identified CNVs that contain genes and are located on 14 chromosomes. The figure presents chromosomes with identified CNVs, participant ID and phenotype group: 1. cryptorchidism, 2. infertility, and 3. infertility and cryptorchidism. We identified a higher number of copy number losses than gains (36 vs 6). Out of the 18 CNVs located on 14 chromosomes, ten chromosomes include one CNV, four chromosomes (1, 2, 5, and 9) include two CNVs, while 10 chromosomes do not contain CNVs. Twelve CNVs are present in only one participant group; namely, seven CNVs were identified only in the cryptorchidism group and six CNVs were identified only in the infertility group. Four CNVs are present in two participant groups, and one CNV is present in all three groups. This result is a confirmation for the multifactorial background of both diseases.



Legend: Red squares represent copy number loss, blue squares represent copy number gain.

Figure 1: Graphical representation of identified CNVs with information on chromosomal locations, presence of genes, sample name, and phenotype group.

Figure 2 shows an example of the aCGH result visualised using the Agilent Cytogenomics software. It represents a 4.68 Mbp CNV loss identified in a participant with cryptorchidism. The analysis software reported that the loss also encompasses the *LRFN5* gene.

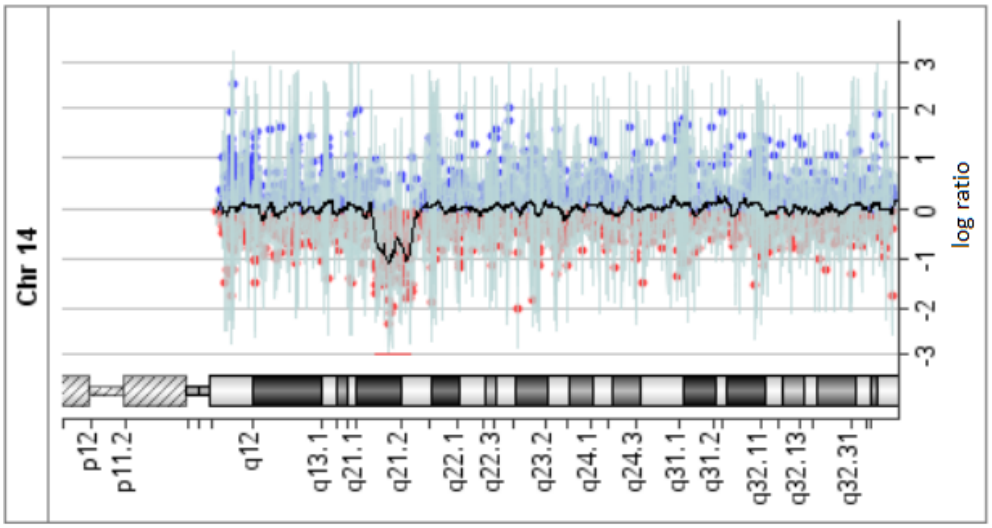


Figure 5: Copy number variation on the chromosome 14 in a patient with cryptorchidism

Regions involved in identified 23 CNVs were analysed with data from the following databases: UCSC genomic browser, DECIPHER, ISCA, OMIM, and PubMed. The 23 CNVs were further characterised on the basis of the following five criteria for association with cryptorchidism and/or infertility: 1. expression in the testes and its corresponding structures, 2. presence in the general population, 3. association with syndromes with cryptorchidism in the clinical picture, 4. published studies that described an association of loci with CO and/or infertility, and 5. presence of regulatory regions and interactions with transcription factors (TF) that could influence adjacent loci with changes of expression and thus causing cryptorchidism and/or infertility.

For example, we identified a 4.68 Mbp CNV in a patient with cryptorchidism and compared this region with information in the databases. Several pathogenic CNVs are reported for the region in the ClinGen database, benign CNVs are also reported in the general population (DGV), however neither are common. The identified CNV is a promising candidate for further research of involvement in cryptorchidism development. Five CNVs that are intergenic present promising candidates for further research as they contain regulative regions that could influence adjacent loci and contribute to development of cryptorchidism and/or infertility.

Consideration of clinical significance of the identified CNVs is an important step in the aCGH analysis. Large CNVs are generally considered as pathogenic variants, however large CNVs can be benign variants, and very small CNVs can be pathogenic. Copy number variants can be considered as pathogenic if they are well documented variants with clear clinical significance in several studies. This group also includes large CNVs that have not yet been published in peer reviewed literature on a completely overlapping region, but are at least partially overlapping with a variant with a well determined pathogenic clinical significance. This group also includes most cytogenetically visible variants (3–5 Mbp). In cases where the variants are not associated with well-defined syndromic loci in the region, consideration of the variant as pathogenic must be done with caution. Copy number variants are considered as variants with uncertain clinical significance (VUS) if they contain genes, but there is no known information of importance of gene dosage for the genes included in the CNV. This group also includes CNVs which are described in several studies and databases that report opposing clinical significance for the variant, upon which the variants should be considered as VUS[12]. In cases

where the CNV encompasses a region smaller than 1 Mbp, the CNVs are suggested to be classified as VUS[13]. Copy number variants can be considered as benign if the variants have been reported in several studies and databases as benign variants. In cases where the CNV is well characterised, or is considered as a common polymorphism, it can be considered as benign. For a CNV to be considered as a common polymorphism it must be documented in at least 1% of the population. It is important to consider copy number gains and losses individually, as copy number gains at a specific region can be benign, while copy number losses of the same region can be pathogenic, and vice versa[12]. Additionally, CNVs that are *de novo* deletions or duplications < 1 Mbp can be considered clinically significant. In cases of larger deletions or duplications, that span > 3 Mbp, the loci can be considered as more reliable markers for development of diseases, as the CNV size allows easier additional experimental validation with other appropriate methods such as quantitative PCR (qPCR) and fluorescent *in situ* hybridisation (FISH) [13].

In conclusion, we identified 23 CNV that are candidate risk factors for cryptorchidism and/or infertility. Five CNVs were intergenic, however 18 encompassed genes. Out of the 18 CNVs, seven were identified only in the participant group with cryptorchidism, six only in the participant group with infertility, four in participant groups with either cryptorchidism, or infertility, and one in all three participant groups (cryptorchidism/infertility/cryptorchidism and infertility). However, it is also important to note that these CNVs are also present in the general population. In the future it would be necessary to identify whether these CNVs are *de novo* variants, validate their clinical significance, as well as perform additional validation of CNVs using qPCR and FISH methods.

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POSTERS – INTERACTION GENOME-ENVIRONMENT

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PHYLOGENY AND VIRULENCE-ASSOCIATED GENES AMONG *Escherichia coli* ISOLATED FROM FECES OF PREGNANT WOMEN WITH GESTATIONAL DIABETES

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THE INFLUENCE OF FOLIC ACID AND 5-METHYLTETRAHYDROFOLATE ON THE METABOLIC ACTIVITY DEPENDING ON CHANGES IN THE FOLATE CYCLE GENES

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POVZETEK

Folat ima pomembno vlogo v številnih presnovnih funkcijah. Zato ni presenetljivo, da so folna kislina in njeni derivati, na primer 5-metiltetrahidrofolat (5-Me-THF) eden izmed najpogosteje uporabljenih prehranskih dopolnil. Redno jemanje folne kisline (400 µg/dan) se priporoča v času načrtovanja nosečnosti in v prvem trimestru z namenom preprečevanja napak nevralne cevi, kar je v skladu s priporočili WHO in CDC. Folna kislina ima kompleksno presnovo, njen učinek pa je odvisen od koncentracije biološko aktivne oblike 5-Me-THF. Da bi ocenili, ali obstajajo razlike v odzivu na folno kislino in 5-Me-THF, smo uporabili človeške limfoblastoidne celične linije (LCL) z različnimi genotipi genov folatnega cikla, to so MTHFR, GNMT, MTRR, MTHFD1, FPGS, DHFR, DNMT3B in SLC19A1. Celice smo gojili v prisotnosti različnih koncentracij (6,25 nM, 12,5 nM, 25 nM, 50 nM, 100 nM) folne kisline ali 5-Me-THF in ocenili njihovo metabolno aktivnost po 72h. Višjo metabolno aktivnost smo opazili pri celicah LCL z dodanimi višjimi koncentracijami folata v primerjavi z nižjimi koncentracijami in pri celicah z dodano biološko aktivno obliko folata (5-Me-THF) v primerjavi s folno kislino. Na metabolno aktivnost so vplivali tudi genotipi MTHFR, MTRR, GNMT, DNMT3B, MTHFD1 in SLC19A1. Pri vrednotenju interakcij med spremenljivkami smo ugotovili, da je bila prisotna interakcija med MTHFR genotipom in učinkovino, ter med MTHFR, MTHFD1, GNMT, DNMT3B genotipom in koncentracijo učinkovine. Opažene razlike v odzivu celic LCL na folno kislino in 5-Me-THF nakazujejo na večjo učinkovitost 5-Me-THF pri izboljšanju metabolne aktivnosti celic v primerjavi s folno kislino.

Ključne besede: folna kislina, 5-Me-THF, metabolna aktivnost, polimorfizmi

ABSTRACT

Folate plays a role in many crucial metabolic functions. Therefore, it is not surprising that folic acid and its derivatives, e.g. 5-methyltetrahydrofolate (5-Me-THF) are among the most widely used food supplements. Taking folic acid (400 µg/day) regularly before becoming pregnant and during the first trimester helps to prevent neural tube defects, according to WHO and CDC. Folic acid has a complex metabolism and its effects depend on concentration of the biologically active form 5-Me-THF. In order to determine whether there is any difference in response to folic acid and 5-Me-THF, we used human lymphoblastoid cell lines (LCLs) with diverse genotypes of folate cycle genes, i.e. MTHFR, GNMT, MTRR, MTHFD1, FPGS, DHFR, DNMT3B and SLC19A1. Cells were cultured in the presence of different concentrations (6.25 nM; 12.5 nM; 25 nM; 50 nM; 100 nM) of folic acid or 5-Me-THF and evaluated for metabolic activity at 72 h. Higher metabolic activity was observed in LCLs treated with higher vs. lower concentrations of folates and in cells treated with biologically active

folate (5-Me-THF) vs. folic acid. Metabolic activity was also influenced by MTHFR, MTRR, GNMT, DNMT3B, MTHFD1 and SLC19A1 genotypes. The evaluation of interactions between the analysed parameters revealed an interaction between folate type and MTHFR genotype as well as interactions between folate concentration and MTHFR, MTHFD1, GNMT and DNMT3B genotypes. Our results indicate certain differences in LCLs´ response to folic acid and 5-Me-THF and suggest that 5-Me-THF might be more efficient in improving cell metabolic activity compared to folic acid.

Key words: folic acid, 5-Me-THF, metabolic activity, polymorphisms

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GUT MICROBIOTA OF PREGNANT WOMEN WITH GESTATIONAL DIABETES AND MACROSOMIA

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POVZETEK

Neravnovesje v sestavi črevesne mikrobiote igra pomembno vlogo pri nastanku številnih bolezni, vključno z debelostjo in sladkorno boleznijo. Gestacijski diabetes je vrsta sladkorne bolezni, ki nastane v času nosečnosti in izzveni po porodu. Presežek glukoze v krvnem obtoku nosečnice pomeni, da bo tudi otrok dobil več glukoze kot običajno. Otrok prične proizvajati višje koncentracije inzulina in shranjevati dodatno energijo v obliki maščobe, kar privede do večje rasti otroka in pojava makrosomije. Makrosomni novorojenčki imajo večjo predispozicijo za razvoj diabetesa tipa 2 in prekomerno težo v adolescenci. Na Oddelku za diabetološko ambulantno dejavnost UKC Ljubljana, smo pridobili kandidatke za vključitev v raziskavo ter analizirali osnovne klinične podatke. Želeli smo analizirati sestavo črevesne mikrobiote nosečnic z gestacijskem diabetesom in debelostjo. Uspešno smo optimizirali izolacijo DNA iz blata dveh nosečnic. Obe nosečnici sta kasneje rodili makrosomnega otroka. V nadaljevanju smo genetski material mikroorganizmov preučili z uporabo 16S metagenomike in sekvenatorja Ion PGM™. Primerjali smo vzorca blata odvzeta v drugem in tretjem trimesečju in podatke analizirali s programom Ion Reporter™. Mikrobioti v dveh časovnih točkah ene posameznice sta se izkazali za manj podobni, kot mikrobioti dveh različnih posameznic v isti časovni točki. Razlike v sestavi črevesne mikrobiote med posameznicama so se povečale v tretjem trimesečju. Znano je, da črevesna mikrobiota vpliva na pojav debelosti in presnovnih bolezni. Z analizo podatkov pa smo delno nakazali tudi njen vpliv na pojav makrosomije, zato bi v nadaljevanju bila smiselna analiza večjega števila vzorcev.

Ključne besede: gestacijski diabetes, debelost, mikrobiota, makrosomija

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ABSTRACT

Imbalance in the composition of gut microbiota plays an important role in the emergence of many diseases, including diabetes and obesity. Gestational diabetes mellitus (GDM) occurs during pregnancy and ceases after childbirth. The excess glucose in the mothers' bloodstream passes

through the placenta into the fetal circulation. The fetus subsequently begins to produce more insulin and stores additional energy in the form of body fat, which results in greater growth of the baby and macrosomia. Macrosomal infants are at an increased risk of becoming overweight at a young age and are more likely to develop type 2 diabetes later in life. At University Medical Center Ljubljana, we included patients with GDM and obesity and clinically evaluated them. We successfully optimized the isolation of DNA from the feces of two pregnant women, who later delivered a macrosomal baby. The genetic material of microorganisms was examined using 16S metagenomics and an Ion PGM™ sequencer. We compared two fecal samples taken in the second and third trimester and analyzed the data with the Ion Reporter™ program. Microbiota at two time points from one individual, proved to be less similar than the microbiota of two different individuals in the same time frame. The differences in intestinal microbiota composition among individuals, increased in the third trimester. It is known that gut microbiota is linked with development of obesity and metabolic diseases, but our analysis could also partially explain its impact on macrosomia occurrence, however, more detailed analysis on a larger number of samples, would be necessary.

Key words: gestational diabetes mellitus, obesity, microbiota, macrosomia

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ANALYSIS OF ANTIBIOTIC RESISTANCES AMONG *Escherichia coli* ISOLATED FROM FECES OF PREGNANT WOMEN WITH GESTATIONAL DIABETES

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IZVLEČEK

Človeška črevesna mikrobiota je pomembna za naše zdravje in ena od najpomembnejših fakultativno anaerobnih vrst v črevesni mikrobioti je *Escherichia coli*. Naš cilj je bil določiti, kako pogosto se v sevih *E. coli* nosečnic z gestacijskim diabetesom pojavljajo določene rezistence proti antibiotikom. V izolatih bakterije *Escherichia coli* iz vzorcev blata nosečnic z gestacijsklim diabetesom, iz drugega in tretjega tromesečja nismo zaznali rezistence proti natrijevemu azidu in ceftazidimu. Vsi sevi nosečnic z makrosomnim otrokom v drugem tromesečju so bili rezistentni proti kanamicinu, vendar ja ta delež v tretjem tromesečju padel. Rezistenci proti kloramfenikolu in ciprofloksacinu smo zaznali le pri sevih nosečnic z makrosomnim otrokom iz drugega tromesečja. V naših vzorcih je bil le en sev pozitiven za *qnrB*, prav tako so bili vsi negativni za CTX-M. Naši rezultati nakazujejo, da obstajajo razlike v razistencah proti antibiotikom med sevi nosečnic, ki so rodile makrosomnega otroka in tistimi, ki so rodile otroka z normalno porodno težo, vendar so potrebne še študije z večjim številom vzorcev.

Ključne besede: *Escherichia coli*, makrosomija, rezistence proti antibiotikom

ABSTRACT

Human gut microbiota plays a an important role in our health and one of the most important facultative anaerobic species in the gut microbiota is *Escherichia coli*. Our aim was to determine the occurrence of certain antibiotic resistances in *E. coli* strains from preagnant women with gestational diabetes. No resistance to sodium azide and ceftazidime was observed among *Escherichia coli* isolates from fecal samples of pregnant women with gestational diabetes, in the second and third trimester. All strains from women with macrosomic babies in the 2nd trimester were resistant to kanamycine but in the 3rd the number dropped. Only strains from women with a macrosomic baby and in the 2nd trimester were resistant to chloramphenicol and ciprofloxacin. Only one strain was positive for *qnrB* while all examined strains were negative for CTX-M. Our findings indicate certain differences in antibiotic resistances between strains from women who gave birth to a macrosomic baby and women with normal sized babies, but studies with a larger sample group will be needed.

Key words: *Escherichia coli*, fetal macrosomia, antibiotic resistances.

INTRODUCTION

The intestinal microbiota is the largest microbial ecosystem of human microbiome and is very important for biological processes throughout the human lifetime (Secher, 2016). Emerging evidences

show that the human microbiota is intrinsically linked with overall health. It is recognized to play vital roles in maintaining host health and has a profound effect on human diseases, including obesity, diabetes, cardiovascular diseases, inflammatory bowel diseases, cancers and other diseases (Xu, 2015). One of the first colonizers of the gut after birth is the facultative anaerobe *Escherichia coli*, its diverse population includes harmless commensal, probiotic and deadly pathogenic strains (Secher, 2016).

Fetal macrosomia is a term used to define newborns who are significantly larger than average (birth weight ≥ 4,000 g). Between 15 and 45% of newborns of mothers with gestational diabetes mellitus are macrosomic (in comparison to 12% of newborns of normal mothers). Macrosomic babies also have a higher risk of becoming overweight or obese at a young age and are more likely to develop type ll diabetes later in life (KC, 2015).

Antibiotic resistance is a major, global health care and community problem. The number of resistant strains is increasing among bacterial pathogens and is reaching alarming levels (Collignon, 2015). In February 2017, the World health organization (WHO) published a "Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics" (WHO, 2017). The scope of the WHO publication was to identify the most important resistant bacteria at a global level for which there is an urgent need for new treatments. On this list are, among the most critical, with priority number 1, the *Enterobacteriaceae* including *E. coli*. Human microbiota also plays a major role as natural reservoir of bacterial resistance to antibiotics. Fluoroquinolones (FQ) are among the most prescribed antibiotics and as a direct consequence a major increase in FQ resistance is occurring worldwide. Emergence of FQ resistance also further promotes the emergence of multiresistant bacteria. Described have been plasmidic resistance mechanisms, which confer low-level resistance to FQ and their accumulation in a step-by-step fashion leads to high-level resistant strains (de Lastours, 2015).

Three different mechanisms of plasmid-mediated quinolone resistance (PMQR) have been determined: target protection (Qnr proteins), active efflux pumps and drug modification. The three mechanisms alone cause only low-level resistance to quinolones, but they can complement other mechanisms of chromosomal resistance to reach clinical resistance level and facilitate the selection of higher-level resistance. PMQR genes are usually associated with mobile or transposable elements on plasmids, thus they can easy spread via horizontal gene transfer. Six major Qnr families have been identified: QnrA, QnrB, QnrS, QnrC, QnrD and QnrVC (Rodríguez-Martínez, 2016).

Production of extended-spectrum β-lactamases (ESBLs) is the principal mechanism of resistance to oxyimino-cephalosporins evolved by members of the family *Enterobacteriaceae*. Several types of CTX-M enzymes can be found among clinical pathogens. The CTX-M-type β-lactamases include at least six sublineages of groups CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25 and KLUC, there are also at least four CTX-M variants that exhibit a hybrid structure. The majority of variants are found within the CTX-M groups 1 and 9, suggesting a higher plasticity. Acquired $bla_{CTX\text{-}M}$ genes are generally carried by conjugative plasmids, although in some strains they were found integrated into the chromosome. The association of CTX-M-encoding plasmids with highly successful virulent clonal lineages has generated a number of the so-called "high-risk" multiresistant and virulent clones (D`Andrea, 2013).

MATERIAL AND METHODS

Bacterial strains. The used *E. coli* isolates were isolated on MacConkey plates from feces of pregnant women in the period between March and July 2017. Each woman gave two samples, one in 2nd and one in 3rd trimester.

Resistance profile. Isolated strains were first grown on LB plates overnight at 37 °C. Next day they were transferred on selection plates by sterile velvet. Selection plates were LB plates supplemented with one of the following antibiotics: ampicillin (100 µg/mL), tetracycline (10 µg/mL), streptomycin (150 µg/mL), sodium azide (160 µg/mL), chloramphenicol (20 µg/mL), kanamycin (50 µg/mL), ceftriaxone (2 µg/mL), nalidixic acid (25 µg/mL), ciprofloxacin (1,3 µg/mL) or ceftazidime (30 µg/mL). Plates were incubated overnight on 37°C. A resistance profile was determined for each isolate and used to assume the number of different strains in each fecal sample. We also determined how often a certain resistance occurred in 2nd and 3rd trimester, the difference in number of strains and resistances in samples from women with macrosomic or normal baby.

Genotypization for CTX-M and *qnr*. A multiplex PCR for *qnrA, qnrB* and *qnrS* was made for pools of 5 lysates using MasterMix (Thermo Fisher Scientific). We used primers and protocol as described by Cattoir *et al.* (2008). Lysates from positive pools were individually retested. In case needed, the PCR specificity was determined with two different primer annealing temperatures (54 °C and 58 °C). Also, the CTX-M was assayed in pools of 5 lysates with MasterMix. In the case of CTX-M, we used primers and protocol as described by Woodford (2010). Lysates from positive pools were individually retested.

RESULTS AND DISCUSSION

Assumed number of *E. coli* strains. Based on the numbers of determined resistance profiles we can assume that in the 2nd trimester most women had only one *E. coli* strain. In the 3rd trimester most women had one or two different strains. The highest number of different strains was 6 and it was found in 10% of analyzed 3rd trimester samples. There was no big difference in number of strains between 2nd and 3rd trimester (Figure 1).

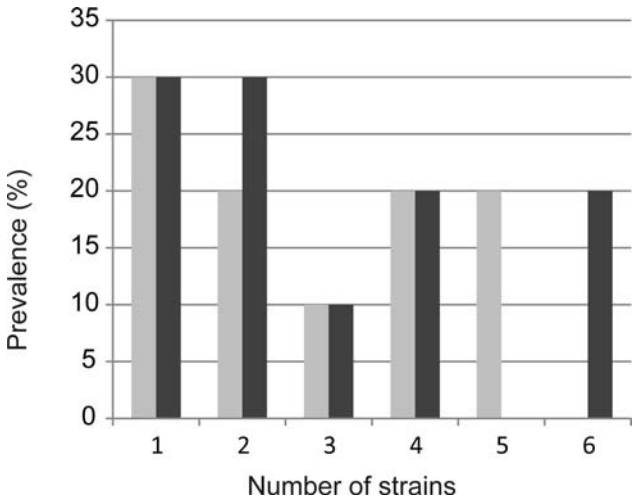

Figure 1: Prevalence of women (in %) with certain number of strains in 2nd and 3rd trimester. N=10. Data of women from whom we had samples from both trimesters are shown. Data from samples of 2nd trimester are in light grey, data from samples in 3rd trimester are in dark grey.

For 7 women we could obtain the data about the birth weight of the baby. A separate analysis of the resistance profiles for them was done. Women who gave birth to a macrosomic baby had more different strains of *E. coli* in 2nd trimester (two, four or five strains) than in 3rd (one, two or four strains). Other women had more strains in 3rd trimester (two, three, four of six strains) than in

2nd (one, two, four or five strains). Loss of *E. coli* diversity in the last trimester could be linked to fetal macrosomia.

Antibiotic resistances: In 2nd trimester the most common antibiotic resistance was the resistance to kanamycin (80%), followed by tetracycline and streptomycin resistance (60%). In 3rd trimester the most common resistance was to kanamycin and streptomycin (50%). In 2nd trimester there were more resistances present than in 3rd and the percentage was also higher (except with nalidixic acid). Resistances to sodium azide and ceftazidime were not present at all.

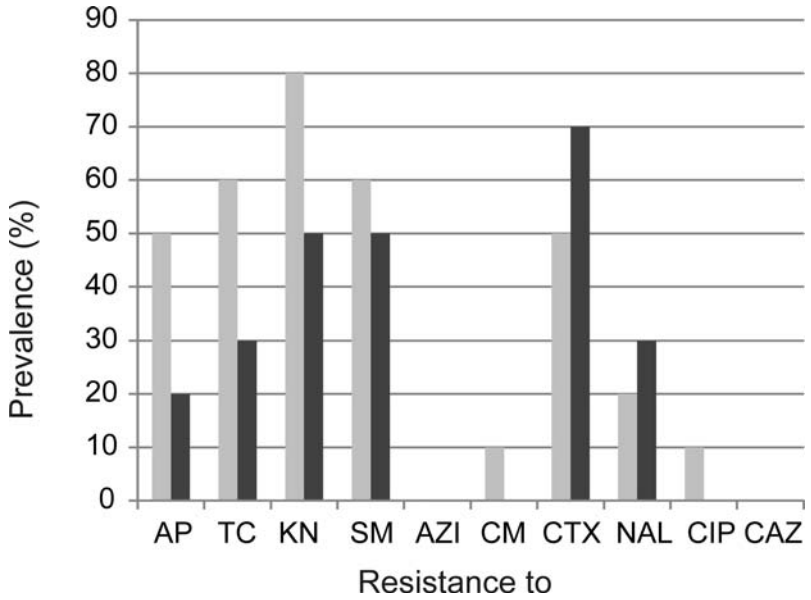

Figure 2: Prevalence of women (in %) with certain resistance to antibiotic. N=10. Data of women from whom we had samples from both trimesters are shown. Data from samples of 2nd trimester are in light grey, data from samples in 3rd trimester are in dark grey. AP – ampicilin, TC – tetracyclin, KN – kanamycin, SM – streptomycin, AZI – sodium azide, CM – chloramphenicol, CTX – ceftriaxone, NAL – nalidixic acid (25 µg/mL), CIP –ciprofloxacin, CAZ–ceftazidime.

Most common resistances in samples from women who gave birth to a macrosomic baby in 2nd trimester were resistance to kanamycine (100%), ampicilin, tetracycline and streptomycine (66%). In 3rd trimester the most common resistances were to tetracycline, streptomycine and nalidixic acid (66%). In samples from women with normal sized babys in 2nd trimester the most common resistance was to kanamycin (75%) and in 3rd trimester to kanamycin and streptomycin (75%).

No resistance to sodium azide and ceftazidime was observed. All strains (100%) from women with macrosomic baby in 2nd trimester were resistant to kanamycin, but in 3rd only 33% of strains had kanamycin resistance. While women with normal baby had in both trimesters 75% of strains resistant to kanamycin. Only strains from women with macrosomic baby in 2nd trimester were resistant to chloramphenicol and ciprofloxacin (33%). Among women who had a macrocomic baby 33% of strains were resistant to nalidixic acid in 2nd and 66% in 3rd trimester, but in strains from women with normal baby the number stayed low (25%).

Genotypization for *qnr* and CTX-M: All samples were negative for *qnrA* and *qnrS*, for *qnrB* only one sample was positive (it was also resistant to streptomycin). Two individually checked samples seemed *qnrS* positive and were retested with two different primer annealing temperatures

(54 °C and 58 °C) to determine, if the PCR product was specific or nonspecific. As at 58 °C we could not re-obtain the PCR product that was amplified at 54°C of annealing temperature, we concluded that binding of primers at 54 °C was nonspecific and thus both strains were actually *qnrS* negative. One strain was positive for *qnrB*. All samples were negative for all CTX-M. Since all samples were negative for CTX-M, there is no resistance to CTX, even though we observed an unusual growth on CTX agar plates.

Although our study revealed differences in antibiotic resistance prevalence, further studies with a much larger number of samples will be needed to enable us to make some definite conclusions.

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THE PREVALENCE OF GENOTOXINS (Usp and Clb) AND CLONAL DIVERSITY ANALYSIS OF *ESCHERICHIA COLI* ISOLATES FROM WOMEN WITH GESTATIONAL DIABETES

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IZVLEČEK

Eden izmed načinov za ugotavljanje raznolikosti bakterije in bakterijske združbe *Escherichia coli* (*E. coli*), izolirane iz fecesa žensk z gestacijskim diabetesom, je določitev števila različnih klonov z uporabo metode ERIC-PCR in z opazovanjem prisotnosti genskih zapisov za dejavnike virulence (genotoksini). Pri večini nosečnic (41%) je bil v blatu prisoten le en klon fekalne *E. coli*. Iz posameznih prostovoljk smo jih izolirali največ pet. Odstotek žensk s petimi različnimi kloni *E. coli* se je zvišal z 8 % v drugem na 25 % v tretjem trimestru nosečnosti. Opažena je bila tudi razlika med suhimi in debelimi ženskami. Pri debelih nosečnicah se je število različnih klonov *E. coli* v tretjem trimestru spremenilo, pri normalno težkih in suhih nosečnicah pa ni bilo opazne razlike med vzorčenjem v drugem in tretjem trimestru. Test za prisotnost dveh genotoksinov (*usp* in *clb*) pri fekalni *E. coli* je razkril veliko večjo razširjenost gena *clb* v primerjavi z genom *usp,* ki smo ga zasledili le pri enem izolatu. Opazili smo, da se gena *clbA* in *clbS* v večini primerov pojavljala skupaj, saj je bilo zelo malo sevov, ki so imeli le *clbA* ali *clbS.*

Ključne besede: *E. coli*, nosečnost, diabetes, ERIC-PCR, genotoksin

ABSTRACT

A way of studying bacterial diversity of *E. coli*) isolated from feces of women with gestational diabetes is by determining the number of clones using ERIC-PCR method and by observing the presence of virulence factor genes (e.g. genotoxins). While the highest number of clones detected in one woman was five, most of them (41%) expressed only one clone of fecal *E. coli.* The percentage of women with five clones of *E. coli* raised from 8% in the second to 25% in third trimester. The difference was also observed among slim and overweight women. The latter showed a change in the number of clones in the third trimester while no difference between semesters was observed among slim women. Out of the two genotoxin genes that were amplified in *E. coli* strains *clb* was more common than *usp,* being detected only in one clone. We also observed that genes *clbA* and *clbS* commonly appeared together, since very few strains showed the presence of either *clbA* or *clbS*, respectively.

Key words: *E. coli*, pregnancy, diabetes, ERIC-PCR, genotoxin

INTRODUCTION

Escherichia coli (*E. coli*) is a commensal bacterium, normally residing in the lower part of the gastrointestinal tract of warm blooded animals, including humans. *E. coli* belongs to the normal gut microbiota and it contributes to normal function of human body. With its human host the bacterium

is in a mutualistic relationship that means that both, the host and the bacterium *E. coli,* are having benefit. Host`s digestive system provides the bacterium a warm, moist and nutrient rich environment while *E. coli* contributes to its host`s health by, for example, producing the vitamin K and by using up the present oxygen, thus enabling other anaerobic microorganisms to survive (Madigan, 2015).

It is known that human intestine microbiome can affect insulin resistance and overall metabolic health (Kuang, 2017). Since *E. coli* is a part of normal human gut microbiota, this study aims to find any possible connection between the onset of gestation diabetes and *E. coli*.

Gestation diabetes is a health condition in which otherwise healthy women develop high levels of sugar in their blood during pregnancy. It appears during pregnancy and disappears after the delivery (Serlin, 2009).

The aim of our study was to find a possible connection between the characteristics of microbiota, with the focus on *E. coli,* and gestational diabetes. We isolated different strains of *E. coli* from fecal samples taken during the second and the third trimester of pregnancy. Isolated *E. coli* strains were then subjected to characterization using PCR based techniques.

A way of characterizing *E. coli* strain diversity is the use of ERIC-PCR method. Enterobacterial repetitive intergenic consensus (ERIC) sequences with a size of 127 bp are present in genomes of enteric bacteria and occur in multiple copies. Some ERIC sequences are highly conserved while other are variable, which allows us to distinguish between different clones among isolates (Wilson, 2006). The sizes of ERIC-PCR products varies between 150 -2000 bp, resulting in distinct patterns, the so called ERIC-profiles, after electrophoresis on agarose gels (Versalovic, 1991).

After establishing clonal distribution of ERIC sequences, using PCR, non-clonal isolates were assigned to phylogenetic groups and subgroups using PCR according to Clermont et al. (2000). Subsequently, we observed the presence of various virulent factor genes, including two genotoxins (*usp* and *clb)*.

Bacterial genotoxins are chemical agents that damage DNA molecules in a cell and in that way cause mutations which are further on responsible for cell death. So far, they have been found in strains of *E. coli*, *Haemophilus ducreyi*, *Shigella dysenteriae*, *Campylobacter* sp., *Helicobacter* sp., *Salmonella enterica* serovar Typhi etc. Usp and Clb are commonly found among pathogenic *E. coli* of phylogenetic group B2 and D and act as important virulence factors. The DNA damage can be induced either by single or double strand breaks. The cell expressing the genotoxin is usually protected from its effect by producing proteins that neutralize the effect of the genotoxin. Both genotoxins can be found in pathogenic *E. coli* (Grasso, 2015). Uropathogenic-specific protein (Usp) was first discovered in uropathogenic *E. coli* isolates and is commonly associated with Imu1-3 proteins that assure the cell producing Usp is protected. Colibactin (Clb), on the other hand, is a product of a gene cluster including, among others – *clbA*, *clbQ* and *clbS* , that reside on a pathogenicity island (PAI) and promotes carcinogenesis in mammalian cells (Nešeć, 2004).

MATERIALS AND METHODS

E. coli were isolated from the feces of pregnant women in different time periods (during the second and the third trimester of pregnancy). The bacterial lysates were obtained from overnight bacterial cultures (~10^9 cells/ml) by resuspending pelleted cells in sterile distilled water, followed by boiling for 10 min. After centrifugation supernatant containing bacterial DNA was stored at – 80 °C.

The presence of genotoxins was determined by PCR, in PCR reactions as follows: each PCR mix included 2.5 µL of PCR Buffer, 0.2 µL DNA polymerase, 0.5 µL dNTP, 0.5 µL of each primer (10 pmol/µl), 1 µL bacterial lysate and 20.3 µL dH_2O. The initial denaturation of DNA was at 94 ˚C for 4

min, followed by 30 cycles of denaturation at 94˚C for 30 s, primer annealing at 55 ˚C for 30 s and elongation at 72 ˚C for either 60 s (*clbAQ*), 40 s (*clbS*) or 90 s (*usp*), and a final elongation at 72 ˚C for 7 min. Primers for detecting *clbAQ* and *usp* were as published in Johnson et al. (2008) and Vadnov et al. (2017), respectively. Primers for detecting *clbS* gene were: 5'-TTACGCGTTA-TTCTGCAAGACATTTCTGCA-3' (forward primer) and 5'- TTGGATCCGCTGTTCCATCATCAAAAGAAGAG-3' (reverse primer). The PCR products were analyzed by gel electrophoresis on 1.5 % agaroses gels (120 V, 40 min).

The PCR mix for ERIC-PCR contained 12.5 µL DreamTaq PCR Mix, 9.5 µL dH_2O, 1 µL of each primer and 1 µL bacterial lysate. The initial denaturation of DNA was at 94 ˚C for 4 min, followed by 30 cycles of denaturation at 94 ˚C for 30 s, primer annealing at 40 ˚C for 15 s, elongation at 72 ˚C for 4 min and a final elongation at 72 ˚C for 7 min (Varsalovic, 1991). PCR products were analyzed as described above.

RESULTS AND DISCUSSION

Diversity of *E. coli* strains was determined with ERIC-PCR method based on distinctive profiles of *E. coli* strains. ERIC-profiles were determined and recognized as a characteristic pattern of bands formed by the number of ERIC-PCR products of varied sizes on electrophoretic gel.

After examination of ERIC-profiles of 404 isolates collected from feces of 17 women with gestational diabetes we determined that the highest number of different strains detected, in both trimesters, in any out of the 17 women, was five. Moreover, it was observed that 41% of women had only one distinctive *E. coli* strain in at least one of the two trimesters. The prevalence of the number of different strains within the second trimester was as follows: 33% of women had only one distinctive strain, 25% of women had two, equal percentage (17%) of women had 3 and 4 different strains respectively, while 8% of women had five different *E. coli* strains. The prevalence of the number in the third trimester differed, since it was observed that 50% of women had only one distinctive strain, no women had two strains, equal percentage (13%) of women had 3 and 4 different *E. coli* strains, respectively, while the highest number (5) of different *E. coli* strains was observed in 25% of women included in this research.

A comparison between the numbers of different *E. coli* strains isolated from the same person in both two trimesters was also done. In the case of two slim women, the number of different *E. coli* strains in two different trimesters was as follows: the first woman had in both trimesters only one distinctive strain of *E. coli*, while the second woman had 3 different strains in the second trimester and 4 different *E. coli* strains in the third.

Comparison between two slim women and one fat woman revealed considerable difference in the number of different strains between two trimesters. In one fat woman we isolated one *E. coli* strain in the second trimester and five strains in the second one.

On the basis of our preliminary results we conclude that among slim women in the second trimester the number of different strains was in favor of lesser number of *E. coli* strains, while in the third trimester we have an ambiguous situation with half of the female participants having just one distinctive clone, whereas the other half had predominantly higher number of different strains. It is noticeable that in both, the second and the third trimester, the characteristic number of different *E. coli* strains was one. In the case of one fat woman there is a huge difference in the number of different strains between the two trimesters, as we isolated one *E. coli* strain in the second trimester and five strains in the second one.

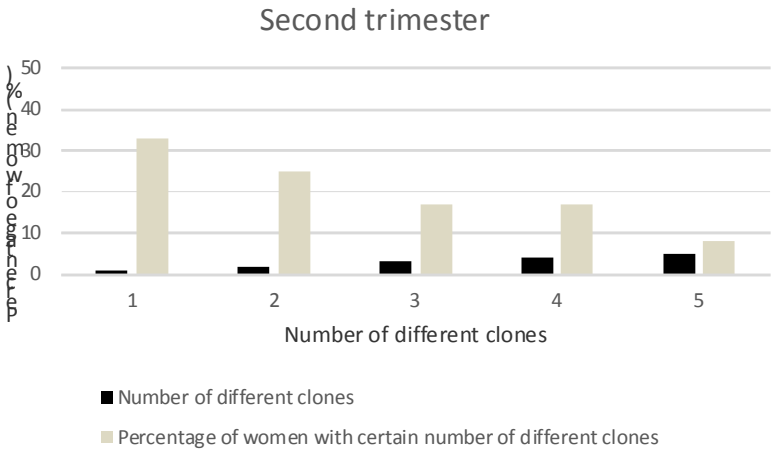

Figure1: Graphical presentation of the number of different bacterial clones in the second trimester of pregnancy among women with gestational diabetes.

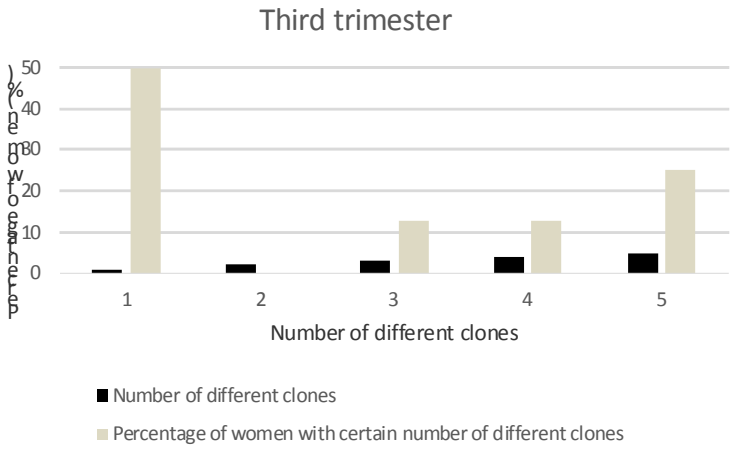

Figure 2: Graphical presentation of the number of different bacterial clones in the third trimester of pregnancy among women with gestational diabetes.

Table 1: Comparison between the number of different clones in two trimesters in the same woman.

		Number of different bacterial clones
Slim woman 1	2. trimester	**1**
	3. trimester	**1**
Slim woman 2	2. trimester	**3**
	3. trimester	**4**
Fat woman	2. trimester	**1**
	3. trimester	**5**

PCR was performed for the presence of *clbS* on *E. coli* isolates that were earlier found to be positive for the *clbAQ*. We found that 18 out of 22 *clbAQ* positive isolates (82%) were also positive for *clbS*. Having in mind the colibactin pathogenicity island and its structure it was expected that all of the isolates were either tested positive for *clbAQ* and *clbS* or for none of them. In order to understand, why in 4 strains we could not amplify the *clbS*, further tests, including whole pathogenicity island sequencing, are to be performed.

IIA30 and IIB11 isolates were also tested for the presence of *clbS* and *usp*. Whereas PCR showed no positive results for the *clbS*, we successfully amplified the *usp* gene only in IIA30/1.

Table 2: Results of PCR amplification of *clbA*, *clbS* and *usp* genes.

***E. coli* isolate**	***clbA***	***clbS***	***usp***
TI 1	–	–	/
TI 2	–	–	/
TI 8	+	–	/
TI 16	–	–	/
TI 17	–	–	/
TI 18	–	–	/
TI 20	–	–	/
TI 22	–	–	/
TI 25	–	–	/
TI 29	–	–	/
TI 32	+	–	/
TI 33	+	–	/
TI 35	–	–	/
TI 36	–	–	/
TI 37	+	+	/
TI 38	+	+	/
TI 39	+	+	/
TI 40	+	+	/
TI 41	+	+	/
TI 42	+	+	/
TI 45	+	+	/
TI 49	+	–	/
IIA30/1	/	–	+
IIB11	/	–	–

/ - no data

Presence of certain virulence factors among *E. coli* strains from normal gut microbiota is not surprising, as gut microbiota is considered to be the potential pool from which new pathogenic strains of *E. coli* can be recruited. Further, it is known that otherwise commensal strains, if they are transferred to other/new environments (e.g. blood, urogenital tract …) and put under different selection pressures can exhibit their hidden virulence potential.

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PHYLOGENY AND VIRULENCE-ASSOCIATED GENES AMONG *Escherichia coli* ISOLATED FROM FECES OF PREGNANT WOMEN WITH GESTATIONAL DIABETES

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IZVLEČEK

Bakterija *Escherichia coli* sodi med najbolj preučevane mikroorganizme. Je običajno komenzalna bakterija, a nekateri sevi te vrste so tekom evolucije pridobili genske zapise za virulentne dejavnike in tako zmožnost povzročitve bolezni. Genski sklad *E. coli* je zelo bogat in sevi *E. coli* iz različnih okolij se med sabo lahko precej razlikujejo. S filogenetsko analizo in preverjanjem razširjenosti posameznih, z virulenco povezanih genov, smo v naši raziskavi poskušali ovrednotiti pestrost sevov *E. coli*, izoliranih iz blata nosečnic z gestacijskim diabetesom, saj o tem v literaturi ni podatkov. Med 56 preučevanimi sevi smo tako ugotovili, da je približno četrtina sevov iz filogenetske skupine B2, ki je povezana s patogenimi sevi *E. coli*. Prevalence preučevanih z virulenco povezanih genov pa so bile naslednje: *fimH* (77 %), *papGIII* (16 %), *sfaDE* (16 %), *afa/draBC* (4 %), *iha* (23 %), *hlyA* (16 %), *clbAQ* (18 %), *cnf1* (18 %) in *iucD* (20 %). Prisotnost gena *ireA* nismo zaznali v nobenem preučevanem sevu.

Ključne besede: *Escherichia coli*, filogenija, z virulenco povezani geni, gestacijski diabetes

ABSTRACT

The bacterium *Escherichia coli* belongs to the most studied microorganisms. Usually it is a commensal bacterium, however during evolution some of the strains have obtained genes encoding virulence factors, and thus gained pathogenicity. The *E. coli* gene pool is very rich and *E. coli* strains from different environments can be very diverse. The aim of our study was to investigate, by determination of the phylogenetic group and prevalence of virulence-associate genes, the genetic diversity of *E. coli* strains isolated from feces of pregnant women with gestational diabetes, as to our knowledge in the literature there are no data about it. Among the 56 studied strains, approximately one quarter belonged to the phylogenetic group B2, which is associated with pathogenic strains. The prevalences of the virulence-associated genes were as follows: *fimH* (77%), *papGIII* (16%), *sfaDE* (16%), *afa/draBC* (4%), *iha* (23%), *hlyA* (16%), *clbAQ* (18%), *cnf1* (18%), and *iucD* (20%). The *ireA* gene could not be found in any studied strain.

Key words: *Escherichia coli*, phylogeny, virulence-associated gene, gestational diabetes

INTRODUCTION

The bacterium *Escherichia coli* is Gram negative and a member of the *Enterobacteriaceae* family. It is the primary inhabitant of the vertebrate gut and colonizes infant's gut within hours after

birth (Kaper, 2004; Crossman, 2010). Thereafter, for the remainder of life, *E. coli* is the most abundant facultative anaerobe of human microbiota and is living in symbiosis with its host (Tenaillon, 2010).

However, throughout evolution some of the *E. coli* strains have acquired specific virulence factors and are therefore able to provoke various intestinal (IPEC-intestinal pathogenic *E. coli* strains) and extraintestinal infections (ExPEC-extraintestinal pathogenic *E. coli* strains), such as urinary tract infections, meningitis, pneumonia, skin and soft-tissue infections and sepsis. Specific virulence factors include various toxins, adhesins, factors to avoid host defense systems and mechanisms for nutrient, especially iron acquisition (Köhler, 2011).

Phylogenetic analysis has shown that *E. coli* strains can be classified into different phylogenetic groups. Members of these groups differ in their ecological niches, life-history characteristics and tendency to cause disease (Tenaillon, 2010). Based on an older classification there were four main phylogenetic groups; A, B1, B2 and D. Most commensal strains were assigned to group A, group B1 consisted of an assortment of different pathotypes and commensal strains. However, ExPEC strains mostly belong to groups B2 and D, members of which diverged simultaneously early in *E. coli* evolution (Leimbach, 2013). Growing genome data for different *E.coli* strains showed an extensive substructure among species. Therefore, a new method had been developed and as a result, now with PCR seven main phylogenetic groups known; A, B1, B2, C, D, E and F can be distinguished (Clermont, 2013). For determination of the phylogenetic group by PCR hence two methods exist: the so called triplex method (Clermont, 2000) and the so called extended quarduplex method (Clermont, 2013)**.** The triplex method uses and multiplies a combination of two genes; *chuA* (288 bp)*,* the outer-membrane hemin receptor gene, and *yjaA* (211 bp), an uncharacterized protein and an aditional DNA fragment, TSPE4.C2 (152 bp), that has recently been identified as part of a putative lipase esterase gene71 (Tenaillon, 2010). This way, *E.coli* strains can be assigned to phylogenetic groups A, B1, B2 and D using a dichotomous key approach based on the presence or absence of the two genes; *chuA*, *yjaA*, and the TSPE4.C2 DNA fragment (Clermont, 2000). The extended quadruplex method is based on triplex PCR method, however, it multiplies a supplementary *arpA* gene (400 bp), which encodes ankyrin repeat protein A. Like the triplex method, the quadruplex method results allow the strains to be assigned to phylogenetic groups A, B1, B2, C, D, E, F and clonal group I using a dichotomous key based on the presence or absence of the three genes; *arpA, chuA, yjaA*, and the TSPE4.C2 DNA fragment. The additional gene, *trpA* (489 bp) is used as an internal control for DNA quality and allows distinction between phylogenetic groups C and E (Clermont, 2013).

Genetic diversity of *E. coli* is very big. The core genome consists of 2.200 genes and the complete gene pool includes more than 18.000 genes (Leimbach, 2013; Tenaillon, 2010). *E. coli* strains from different environments can be very diverse. The aim of our study was to investigate, by determination of the phylogenetic group and prevalence of virulence-associated genes, the genetic diversity of *E. coli* strains isolated from feces of pregnant women with gestational diabetes, as to our knowledge in the literature there are no data about it.

Gestational diabetes mellitus is a clinical risk factor associated with fetal macrosomia. It represents 90% of all types of diabetes occurring in pregnancy. The main complication in women, who develop gestational diabetes, is fetal macrosomia. Generally, fetal macrosomia is defined as a birth weight >4000 g or higher (Araujo Júnior, 2017).

MATERIAL AND METHODS

Bacterial strains. *E. coli* strains were isolated from feces of pregnant women with gestational diabetes in 2nd and 3rd trimester of pregnancy. ERIC-PCR method was used to separate non clonal from

clonal *E. coli* strains. 56 different *E. coli* strains were included in this study. All strains were grown either on LB plates or in liquid LB medium at 37 °C. In case of bacterial growth in liquid LB, the incubation was with aeration (180 rpm).

Bacterial cell lysates. In all performed PCR reactions bacterial cell lysates were used as origin of the matrix DNA. Bacterial cell lysates were prefered with the boiling method as published in Le Bouguénec et al. (1992).

Phylogenetic analysis. For determination of the phylogenetic group both PCR methods were used, the triplex method (Clermont, 2000) and the extended quarduplex method (Clermont, 2013)**.** For 20 µl PCR reaction (triplex method) we used 1 µl of each primer (chuA.1, chuA.2, yjaA.1, yjaA.2, TspE4C2.1 and 1 µl TspE4C2.2, all in concentration 20 pmol/µl), 10 µl of 2×ThermoScientific DreamTaq Green PCR Master Mix 10 mM dNTP, 3 µl of bacterial cell lysate and 1 µl of distilled sterile H_2O. For 20 µl reaction (extended quadruplex method) we used 0.5 µl of primers AceK.f (40 pmol/µl) and of ArpA1.r (40 pmol/µl), 0.5 µl of primers chuA.1b, chuA.2, yjaA.1b, yjaA.2b, TspE4C2.1b and TspE4C2.2b (each in concetration 20 pmol/µl), 10 µl of 2×ThermoScientific DreamTaq Green PCR Master Mix 10 mM dNTP, 3 µl of bacterial cell lysate and 3 µl of distilled sterile H_2O. PCR products were separated and analysed using 1.5 % agarose gel electrophoresis and UV-transilumination.

Genotypization for virulence-associated genes *fimH, papGII, papGIII, sfaDE, afa/draBC, iha, hlyA, clbAQ, cnf1, iucD, ireA* using the PCR method.: For 25 µl reaction we used 0.5 µl forward primer (20 pmol/µl), 0.5 µl reverse primer (20 pmol/µl), 12.5 µl 2×ThermoScientific DreamTaq Green PCR Master Mix 10 mM dNTP, 5 µl cell lysate and 6.5 µl distilled H_2O. PCR products were separated and analysed using 1–2% agarose gel electrophoresis (the agarose % in the gel depended on the expected PCR product size) and UV-transilumination.

RESULTS AND DISCUSSION

Phylogenetic groups. Phylogenetic groups of the 56 strains included in this study were determined with the triplex (Clermont, 2000) as well as the extended quadruplex (Clermont, 2013) method. The distribution of phylogenetic groups among the studied strains is given in Table 1.

Table 1. Distribution of 56 studied *E. coli* strains among phylogenetic groups

	Prevalence (N, %)	Prevalence (N, %)
Phylogenetic group	Triplex method	Extended quadruplex method
A	16 (29%)	17 (30%)
B1	2 (4%)	1 (2%)
B2	19 (34%)	19 (34%)
C	n. a.	0
D	19 (34%)	0
E	n. a.	10 (18%)
F	n. a.	0
Clade I/II	n. a.	8 (14%)
Unknown	n. a.	1 (2%)

n.a. – not applicable

As seen from Table 1 results from triplex and extended quadruplex PCR method gave a similar distribution of strains among phylogenetic groups with both methods. However, there are some exceptions, two strains from group B1 and B2 by triplex method were regrouped to group B2 and unknown by quadruplex method. Strains from the phylogenetic group D by triplex method were in

majority regrouped: 10 *E. coli* strains to extended quadruplex group E, 8 strains to clade I/II and 1 strain to group A. The observed regrouping was expected, as already Clermont et al. (2013) in their publication stated that the new method will lead to several reclassifications among the triplex PCR D group.

Virulence-associated genes. The prevalence (in %) of the investigated virulence-associated genes among studied *E. coli* strains is given in Figure 1.

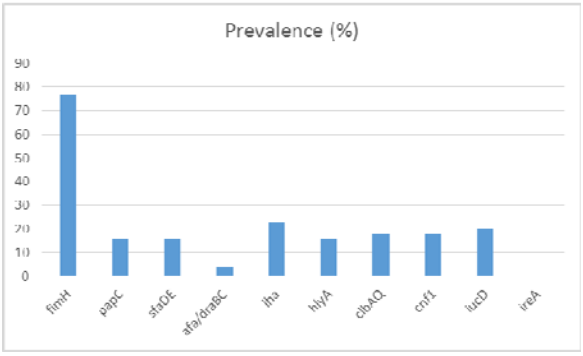

Figure 1: Prevalence (in %) of virulence-associated genes among studied E. coli strains, N=56.

As shown in Figure 1, the most prevalent virulence-associated gene, with 43 positive strains out of 56 tested *E. coli* strains (77%), proved to be the *fimH* gene, which encodes type 1 fimbriae D-mannose specific adhesin. The prevalence of other investigated virulence-associated genes, with the exceptions of *afa/draBC* and *ireA*, where around 20%. IreA gene was not found among the 56 tested strains.

Distribution of virulence-associated genes among phylogenetic groups. An analysis of the distribution of virulence-associated genes among phylogenetic groups, as determined by the extended quadruplex method, was performed. Results are presented in Table 2.

Table 2: Distribution of virulence-associated genes among E. coli phylogenetic groups (based on quadruplex PCR method, Clermont, 2013). The prevalence of virulence-associated genes among phy logenetic groups is given as the total number.

VIRULENCE-ASSOCIATED GENES	PHYLOGENETIC GROUPS BASED ON THE QUADRUPLEX PCR METHOD (CLERMONT, 2013)					
	A	**B1**	**B2**	**CLADE I OR II**	**E**	**U**
fimH	13	0	19	0	10	1
papC	0	0	9	0	0	0
sfaDE	0	0	9	0	0	0
afa/draBC	0	0	1	0	0	1
Iha	2	0	9	0	0	1
hlyA	0	0	9	0	0	0
clbAQ	0	1	7	0	2	0
cnf1	0	1	7	0	2	0
iucD	3	0	8	0	0	0
ireA	0	0	0	0	0	0

Distribution of virulence-associated genes among *E. coli* phylogenetic groups (based on quadruplex PCR method) affirms that genes *fimH, papC, sfaDE, iha, hlyA, iucD, clbAQ, cnf1,*

afa/draBC, are by far most present in B2 phylogenetic group. This was expected, as it is known that the B2 phylogenetic group is associated with pathogenic *E. coli* strains that were isolated from extraintestinal infections (Kohler, 2011; Katouli, 2010). Further, it is known that the reservoir of such extraintestinal pathogenic *E. coli* is the gut microbiota and that in the western human population high levels of B2 strains are expected among the gut microbiota (Nowrouzian, 2010).

In conclusion, from our results we can assume that the *E. coli* strains in the gut microbiota are similar to the *E. coli* strains found in the feces of healthy humans. However, a further study with more included strains from pregnant women with destational diabetes is required to confirm this assumption.

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