Ltd). Phylogenetic trees were constructed using Mr Bayes method with GTR substitution model and the neighbour-joining (NJ) method with Tamura-Nei model of nucleotide substitution.Mean nucleotide distances within (intra-genotype) and among (inter-genotype) BLV genotypes were estimated by adopting the Tamura Nei model in MEGA 4.

Conclusion: All 44 BLV sequences including isolates from Poland, Russia, Ukraine and Belarus were assigned to three genotypes: G4, G7 and a new genotype, G8. We found that most of these isolates (25/44) clustered within G4, fifteen were classified in G7 while four in a new genotype 8. The existence of distinct subgroups within G7 (A, B and C) comprised exclusively of Russian, Ukrainian and Polish isolates, respectively fully corresponded to their geographical origin, since each subgroup contained isolates from one country only. A new genotype, G8 included four isolates from distinct regions of Ukraine and one isolate from Russia. The pair-wise genetic distances analysis within genotype 8 ranged between 0.0–2.3 % and it was relatively lower than those observed for G4 and G7. Surprisingly, this low level of genetic diversity did not reflect the broad geographical origin of these isolates, perhaps indicating the same origin of infection.

Amino acid diversisty of gp51 within BLV isolates. Studying the genetic diversity of viruses could help to gain the correlation between variation in genotype and disease progression, differences in infectivity or potential effect of viral variability on diagnostic assays. We analysed whether nucleotide mutations affected amino acid composition and possibly the conformational structure of envelope glycoprotein gp51.

Amino acid (aa) sequences of the 44 BLV isolates were aligned to aa sequence of BLV-FLK. We noted 21 different amino acids substitutions. Furthermore, although a variety of single aa substitutions were evident over the full length of the analysed part of gp51 some amino acids changes (R121H, H142R, I144T, I176L) were observed in multiple samples. These substitutions encompassed mainly the C-part of CD4+ epitope, zinc binding peptide region, CD8+ T cell epitope and overlapping linear epitope E. We noted the highest numbers of aa substitutions in isolates belonging to G4.

Conclusion. It was interesting that more than half (71%) of all aa substitutions have not been described previously. The biological significance of these changes is unknown, although the histidine replacement (H) by arginine (R) at position 142 could be speculated. In some BLV isolates this histidine was replaced by tyrosine (Y) or leucine (L) [20] and it was shown that this histidine is one of the three histidine residues which are present in the zinc-binding region, which is an essential component of zinc-binding proteins together with cysteine. Taking into account that the region of SU localised between residues 137–156 affects fusion and infectivity of BLV *in vivo* this mutation may be crucial for virus infectivity. Also, the substitution phenylalanine to serine at position 146, found in one isolate, shows that this substitution is exposed on the surface of the second neutralizing epitope and it would diminish immunoreactivity of this epitope.

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МОЛЕКУЛЯРНИЙ АНАЛІЗ ІЗОЛЯТІВ ВІРУСУ ЛЕЙКОЗУ ВРХ – ПОСИЛАННЯ НА ЕПІДЕМІОЛОГІЧНІ ДОСЛІДЖЕННЯ

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У статті наведені дані щодо дослідження 44 ізолятів вірусу лейкозу, виділених з різних географічних регіонів Польщі, Білорусі, України та Росії. Проведено філогенетичний аналіз, за допомогою якого було підтверджено наявність нового генотипу 8.

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PREVALENCE OF ENTEROTOXIN-PRODUCING STRAINS OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM COW'S UDDER IN SERBIA

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Staphylococcus aureus is a major cause of food poisoning, due to the production of heat resistant enterotoxins, which when consumed cause vomiting and diarrhea [1]. Enterotoxins are low-molecular weight proteins (26900–29600 Da). To date, 23 different staphylococcal enterotoxins have been described besides toxic-shock syndrome toxin-1, including staphylococcal enterotoxin A to staphylococcal enterotoxin like V (SEA to SEIV) and they can be divided into five phylogenetic groups [2, 3, 4]. All share superantigenic activity, whereas, only few of them (SEA to SEI, SER, SES, and SET) have been proved to be emetic [4, 5, 6]. SE and SEI have been classified based on their amino acid sequences [3, 4, 7].

Staphylococcal food poisoning is an intoxication that results from the consumption of foods containing sufficient amounts of one or more preformed enterotoxin [8, 9]. Symptoms of staphylococcal food poisoning have a rapid onset (2–8h), and include nausea, violent vomiting, abdominal cramping, with or without diarrhea [10, 11]. The disease is usually self-limiting and typically resolves within 24–48 h after onset. Occasionally it can be severe enough to warrant hospitalization, particularly when infants, elderly or debilitated people are concerned [11].

Staphylococcus aureus is also present in food animals, and dairy cattle, sheep and goats, particularly if affected by subclinical mastitis [12].

In Vojvodina province, 13 (9.77 %) toxin producing *Staphylococcus aureus* strains were found from a total of 133 isolated strains from cow's udders [13]. From Wisconsin dairy herds, 4,2 % of isolated *Staphylococcus aureus* were toxin producing strains [14]. From seven regions of Norway, from different farms, isolates of *Staphylococcus aureus*, collected between August and October in 2001, were tested for staphylococcal enterotoxin production (SEA-SED) by reversed passive latex agglutination and for SE genes (sea–see, seg–sej) by multiplex PCR. *Staphylococcus aureus* was detected in 75 % bulk milk samples. Enterotoxin production was observed in 22,1 % of *S. aureus* isolates, while SE genes were detected in 52,5 % of the bulk milk isolates [15]. In a study of Adwan et al. in the north of Palestine, in 2005, between February and April, a total of 130 raw milk samples were taken from Fresian cows. None of these animals were diagnosed with clinical mastitis. *Staphylococcus aureus* was isolated from 48 (37 %) milk samples. Enterotoxin genes (sea-see) in *Staphylococcus aureus* isolates were determined using a polymerase chain reaction (PCR). Out of 48 *S. aureus* isolates, 14 (29 %) were toxin gene positive, which means that almost 11 % of total milk samples taken were toxin gene positive [16].

Gьcьkoglu et al. published in 2012 results of their investigation on the prevalence of enterotoxigenic *Staphylococcus aureus* isolated from raw milk samples in Samsun province in Turkey. In their study, *S. aureus* was detected in 45 of 60 raw milk samples (75 %). Using multiplex PCR they had determined the presence of genes for the synthesis of staphylococcal enterotoxins SEA, SEB, SEC, and SED, in 13.7% isolates from the raw milk samples [17].

According to The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2006, *Staphylococcus aureus* toxins were responsible for 48,8 % of the 482 human foodborne outbreaks caused by bacterial toxins reported by EU Member States. Staphylococcal enterotoxins were responsible for 243 outbreaks with 2,369 reported human cases and 2 deaths. Dairy products were the known vehicles in 26 (10,7 %) of these outbreaks [18].

In year 2010, regarding to Staphylococcal enterotoxins, 14 EU Member States reported 274 food-borne outbreaks caused by *Staphylococcus* spp., and 13,9 % of these were strong evidence outbreaks with 941 cases of which 20,1 % were hospitalized. There were no case fatalities in 2010 [19]. According to The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011, 15 EU member states reported 345 food-borne outbreaks caused by staphylococcal toxins, representing 6,1 % of all outbreaks reported in the EU. This was an increase of 25,9 % compared with 2010 (274 outbreaks), and was mainly due to the fact that France that reported 290 outbreaks in 2011 compared with 220 in 2010. The overall reporting rate in the EU was 0.07 per 100,000. The highest number of outbreaks was reported by France (84,1 % of the staphylococcal toxins outbreaks), even though, for most of these outbreaks, only weak evidence was provided. One case fatality was reported by France in one weak evidence outbreak. In addition, one non EU-member state reported two outbreaks.

Thirty-five (10,1 %) of the outbreaks were strong evidence outbreaks, distributed fairly evenly among the ten reporting member states. These accounted for 394 cases, of which 27,9 % were hospitalized, but no case fatalities were reported. One strong evidence outbreak due to staphylococcal enterotoxins was reported by Norway. The strong evidence outbreaks caused by staphylococcal toxins was attributed to dairy products in 5,7 % and cheese in 8,6 %.

In Teheran, in 2010, 32 *Staphylococcus aureus* were determined from dairy products: 18 from cream, 10 from cheese, and 4 from milk. They detected each of the toxin gene (SEA, SEB) products by multiplex PCR and compared the results with the phenotypic method. The most frequently contaminated sample by *Staphylococcus aureus* was cream (18 %), and the least frequently contaminated was milk (4 %). Overall, 32 % of the samples were contaminated. The numbers of bacteria in contaminated samples by CFU/mL varied [21].

A diagnosis of staphylococcal food poisoning (vomiting 1-18 hours after consumption of toxic food) is most usually established by the detection of staphylococcal enterotoxin in food consumed by patients. The presence of enterotoxin together with large numbers of organisms in vomitus would also support a diagnosis, although this clinical sample is only very rarely available for analysis. In addition to the presence of enterotoxin, there are usually $>10^6$ CFU/g of an enterotoxin producing *Staphylococcus aureus* present in implicated food. However, because of the stability of staphylococcal enterotoxins which can remain biologically active after cooking and other processes, the toxins can be present in food in the absence of viable organisms, since the latter may be killed during food processing by, for example, cooking or by reduction in pH as occurs during the manufacture of cheese. *Staphylococcus aureus* is intrinsically physically and chemically robust and will tolerate pH ranges from 4,5 to 9,0 and NaCl concentrations up to 9 %. Resistance to heat is dependent upon the surrounding matrices. *Staphylococcus aureus* suspended in 0,9 % NaCl is rapidly inactivated at 46°C, however, when protected by proteins (such as in milk or in pus) it can survive for more than 50 min at 60 °C. [22].

Materials and methods. Our research included highly productive cows that produce over 5,000L of milk per year, from 46 dairy farms in Vojvodina and central Serbia in 2012. These farms have different dairy cows population, from less than 5 cows to over 100 cows. The occurrence of clinical mastitis on these farms was not frequent, but there was increased somatic cell count (over 400,000 SCC/mL) in bulk tank milk samples.

Microbiological analysis of milk samples taken from cow's udder was performed in the Veterinary Specialist Institute "Jagodina". Isolates of *Staphylococcus aureus* had been confirmed using API Staph – bioMйrieux and by PCR detection of nuclease gene.

API Staph is a standardized system for the identification of the genera *Staphylococcus* and *Micrococcus*, which uses a combination of miniaturized standard biochemical tests and fermentation tests which are the reference tests for the identification of staphylococci and a specially adapted database. Identification of staphylococci and micrococci could be done in 18–24 hours. Reliability is guaranteed by a standardized inoculum with a low bacterial concentration avoiding mixed cultures and subcultures. The API Staph strip consists of 20 microtubes containing dehydrated substrates. These microtubes are inoculated with a bacterial suspension, prepared in API Staph Medium that reconstitutes the tests. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to the Reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software.

Enterotoxin production was determined by VIDAS® Set2. It is an immunoenzymatic screening test used for presence of 7 enterotoxins. It is designed for use in automated VIDAS ® system. The test is based on detection of staphylococcal enterotoxin antigens. The detection of staphylococcal enterotoxins consisted of two steps:

1. Extraction/concentration

The sample was mixed and homogenized with distilled water. The toxins which diffused in water were recovered, after two centrifugations, in the supernatant. This aqueous phase was concentrated overnight by dialysis.

2. Immuno-enzymatic detection.

The Vidas SET2 detection is based on an Enzyme Linked Fluorescent Assay (ELFA) test. It is a rapid and fully automated kit detection without differentiation of the staphylococcal enterotoxins types A to E, using a cone coated with antibodies specific for staphylococcal enterotoxins A, B, Cs, D and E (SEA, SEB, SECs, SED and SEE). An immune complex is formed between the coated antibodies, the toxins in the concentrated extract and the anti-SE antibodies conjugated with alkaline phosphatase. All reagents are included in the wells of the strip used. Briefly, the concentrated protein extract was distributed in the strips and incubated in the automate Vidas. Two fluorescence measurements (sample, standard) are performed for each test by the automate and give relative fluorescence values (RFV). The ratio between these two measurements (test value or TV) is interpreted to declare or not a sample as positive.

PCR detection of nuclease gen. Bacterial DNA was extracted from a single *Staphylococcus aureus* colony using 25 µL of nuclease-free water and 25 µL of PrepMan Ultra reagent (Applied Biosystems, Foster City CA, USA) placed in a 1,5 mL micro centrifuge tube. The samples were heated in boiling water for 10 minutes, allowed to cool to room temperature and centrifuged at 16000 Ч g for 2 min. The supernatant (containing the DNA) was transferred to a clean 1,5 mL microcentrifuge tube. PCR amplification of a 255-bp fragment of the nuc gene used primer pair:

• nuc-F (TCAGCAAATGCATCACAAACAG) and

• nuc-R (CGTAAATGCACTTGCTTCAGG).

PCR amplification was performed using a PCR kit (Invitrogen, Carlsbad, CA, USA) in a total volume of 50 µL containing 5 µL of 10Ч reaction buffer, 1 µL of dNTPs, 5 µL of each primers, 1 µL of template DNA, 0,25 µL of Taq DNA (5 U/µL) and 32,75 µL of PCR water to make up the final volume. Amplification was performed using an AB 2720 thermocycler (Applied Biosystems, CA, USA). Thermal cycling conditions was as follows: Initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s with a final extension at 72°C for 5 min. Ten microliters of PCR product were used for electrophoresis in a 1,5 % agarose gel at 120 V for 30 min.

Results and discussion. In our study, using standard microbiological analysis and API Staph bioMйrieux, 75 isolates of *Staphylococcus aureus* have been determined in milk samples from 46 dairy farms.

Enterotoxin-producing strains *of Staphylococcus aureus* are isolated from milk of cows from farms that have different dairy cow population, some as few as 5 cows and some with more than 100 cows. No relationship was noticed between farm size and finding of *Staphylococcus aureus* enterotoxin-producing strains.

In our study, enterotoxin production was determined in 5 out of 75 isolates of *Staphylococcus aureus* (Table 1).

Table 1 – Enterotoxin production via VIDAS® Set2

Sample	TV	RFV	Interpretation
25	2.18	8767	positive
32	2.14	8590	positive
33	2.14	8597	positive
44	1.96	7870	positive
54	1.97	7934	positive
Standard +	--	3916	OK
Standard -	--	4113	OK

TV – Test Value; RFV - Relative Fluorescence Value

6,67 % of *Staphylococcus aureus* is found to be enterotoxin-producing which is much less than findings of other authors. In a study of Adwan et al. in the north of Palestine, in 2005, enterotoxin genes (sea-see) were determined in 29 % *Staphylococcus aureus* isolates using PCR [16]. Gьсьkoglu et al. in Samsun province in Turkey, in 2012, using multiplex PCR had determined the presence of genes for the synthesis of staphylococcal enterotoxins SEA, SEB, SEC, and SED, in 13,7 % of isolates from the raw milk samples [17].

Conclusion. Enterotoxin-production were determined in 6,67 % of *Staphylococcus aureus* strains isolated from cow's udder from 46 dairy farms in Serbia, although it is much less than findings of other authors in other countries, it still indicates their safety risk not only in raw milk, but in the final dairy products due to the staphylococcal enterotoxin thermostability beside the fact that subsequent contamination is often case.

Enterotoxin-producing strains of *Staphylococcus aureus* were determined both on farms that have as few as 5 cows and on farms with more than 100 cows.

Further research is necessary in order to obtain safe milk production and to investigate possible correlation between farm size and finding of *Staphylococcus aureus* enterotoxin-producing strains.

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ПОШИРЕНІСТЬ ЕНТЕРОТОКСИЧНИХ ШТАМІВ *STAPHYLOCOCCUS AUREUS*, ЕКСТРАГОВАНИХ З ВИМ'Я КОРІВ У СЕРБІЇ

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У статті висвітлено результати досліджень щодо поширення ентеротоксичних штамів Staphylococcus aureus, які були екстраговані з вим'я корів. Усього досліджено 46 господарств Сербії та виявлено 6,67 % ентеротоксичних штамів Staphylococcus aureus.