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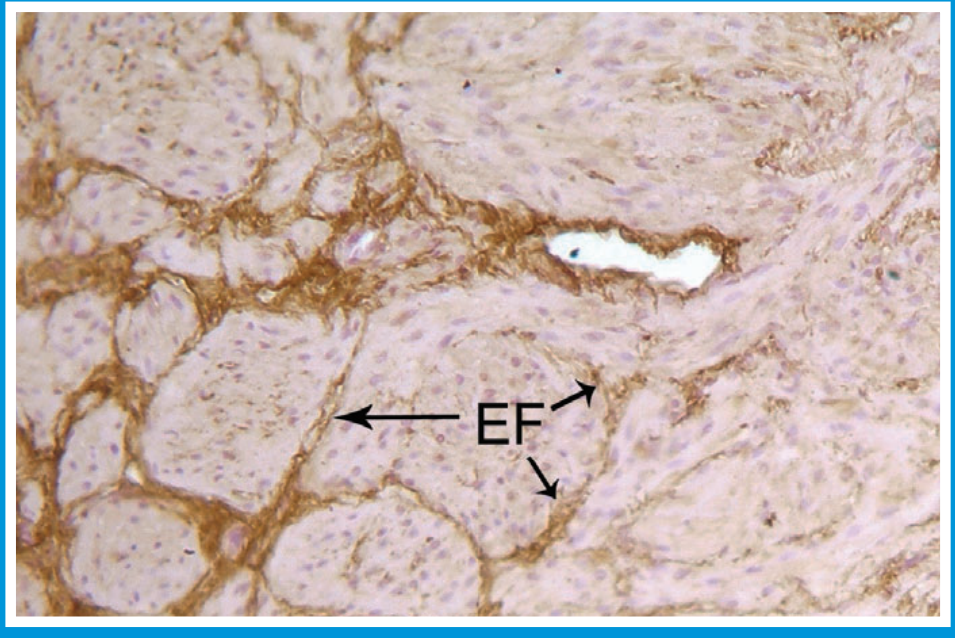

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Original Scientific Article

THE WELFARE ASSESSMENT OF TIED DAIRY COWS IN 52 SMALL FARMS IN NORTH-EASTERN TRANSYLVANIA USING ANIMAL-BASED MEASUREMENTS

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Summary: The aim of this work was to assess the dairy cows' welfare in small farms in the region of North-Eastern Transylvania (Romania) based on several animal associated parameters: body condition score (BCS), body hygiene (cleanliness), lameness, skin injuries, fur condition and flight distance, respectively. Four hundred and seventy three dairy cows housed in tie-stalls in 52 small farms were assessed (5-20 cows/farm). The indicator parameters were determined through specific methods. Among the 473 evaluated cows 267 (56.45%) had a BCS between 1.5 – 2 being considered thin cows; 162 (34.25%) had dull hair on their back; 68 (14.38%) showed skin lesions in different body zones; 69 (14.59%) showed fear at the observer's approach; 23 (4.86%) were moderately lame. The percentage for scores of 3 and 4 in body cleanliness was 11% at the level of the lower legs, 14% in the udder region and 19% in the area of the flank and upper legs. The obtained results indicate that more than half of the assessed cows are thin, which has a negative impact on their health and welfare. The main factors that affect the dairy cows' welfare in North-Eastern Transylvania are inappropriate feeding and the tied housing system.

Key words: body condition; body cleanliness; lameness; skin lesions

Introduction

The dairy cows' welfare represents nowadays a permanent concern in many countries due to its impact on animals' health and productivity and implicitly upon public health. Due to the fact that the dairy cows' welfare has become increasingly related to milk quality, its monitoring constitutes an additional guarantee for the consumers that the products they buy are from healthy animals, maintained and kept in conformity with good practice rules in farming (1).

The economical importance of the animals' welfare assessment lies in the detection of the inadequacies in the first stage and their corrections in the second stage. The farm animals' welfare is provided especially through breeding systems adequate to the health and behavioral needs of the animals as well as through good practices in animal keeping and rearing (2, 3).

Traditionally, farm animals' welfare assessment has focused on the measurement of resources provided to the animal such as housing and its design criteria (5). The use of such indirect resource-based criteria is attractive because their measurement is mostly quick, easy and reliable. Other husbandry aspects that affect animal welfare are management practices and the human-animal relationship. Their measurement is often less easy. However, the provision of good management and environmental resources does not necessarily result in a high standard of welfare. Welfare assessment should therefore primarily be based on animal-related parameters, such as behaviour, body condition score (BCS), body cleanliness, lameness, skin lesions, injuries and swellings, animal-human relationship and so on. Attempts to create an operational welfare assessment protocol primarily relying on animal-related parameters have mainly been made with regard to dairy cows (5-7).

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In Transylvania (Romania) the majority of dairy cows are kept in extensive breeding systems, in small and medium-size farms with tie stalls. It is assumed that the welfare of these animals is better than that of those kept in intensive breeding systems. However, our researches showed that the housing and management of the former has several deficiencies with negative repercussions on the dairy cows' health and welfare (8).

This study's aim was the assessment of dairy cows' welfare kept in tie stalls in small farms in the North-Eastern region of Transylvania, using animal-based indicators.

Materials and methods

The study was conducted at 52 small farms (5-20 cows/farm) in North-Eastern Transylvania, between March and April 2009. The cows' welfare was assessed based on several animal associated indicators, namely: body condition score (BCS), body cleanliness, lameness, skin lesions, fur condition, flight distance. We evaluated 473 dairy cows housed in closed shelters with tie-stalls before the beginning of the grazing season (April-October). During the winter, cows are kept inside the shelters for the whole period and in summer the cows are on the pasture all day long. Each cow was evaluated by two expert examiners. For the assessment of some parameters (lameness, flight distance) the cows were untied and moved outside the shelter.

The body condition score (BCS) was assigned according to the system elaborated by Edmunson et al. (9), modified after Ferguson et al. (10) and Thomsen and Baadsgaard (11) with scores from 1 to 5. Thus, a fat cow is one with BCS≥4; a cow with normal body condition is one with 2.25≤BCS≤3.75; a thin cow is one with 1.5≤BCS≤2 and an emaciated cow is one with BCS≤1.25. For the appraisal of the cow's body cleanliness the scoring system elaborated by Cook et al. (12) was used, which assesses the degree of manure contamination in three body regions: udder, lower and upper leg and flank, awarding points (from 1 to 4). For each area a different score was assigned. At the end, the proportion of scores of 3 and 4 (which means "too dirty") was calculated for the three body regions of the cows. The skin lesions were assessed in all body areas (neck, shoulder and withers, knee, ribs, back/spine, tailbone, hipbone, point of hock, inside of the hock and stifle) through the method proposed by Leeb et al. (13). The lameness was evaluated based on the system elaborated by Sprecher et al. (14). The fur condition was assessed based on the aspect of the hair of the dorsal region of the cow's back: shiny hair; dull hair with little dust on the back; very dull hair with much dust on the back – through the method proposed by Thomsen and Baadsgaard (11). Flight distance (avoidance distance) was appreciated by measuring the distance (in meters) to how close we could approach the cow before it retreats from us (13). In order to determine the avoidance distance, the cows were untied and moved outside the barn. The participant in the test waited at a distance of 3 to 4 m for the cow to look at him before approaching, i.e. more or less directly from the front, walking slowly (one moderate step per second), looking at the cow without direct eye contact, and keeping arms and hands close to the body. We considered that the cows do not show fear when the avoidance distance is smaller or equal to 1 m and that the cows show fear when the avoidance distance was more than 1 m.

After the cows' assessment, the results were expressed as percentages.

Results

The results obtained through the assessment of the 473 cows are shown in Figures 1 - 3. Figure 1 shows that 56.45% of the assessed cows had a BCS between 1.5 and 2, being considered thin cows; 34.25% had dull hair on their back; 14.59% showed fear at the approach of the examiner; 14.38% had skin lesions in different body regions (neck, shoulder, legs); 4.86% were moderately lame.

The percentage of the 3 and 4 scores of body cleanliness at the level of the three evaluated body areas for the 473 assessed cows were: lower legs 11%, udder 14%, flank and upper legs 19% (Figure 2).

The distribution of the BCS scores is shown in Figure 3.

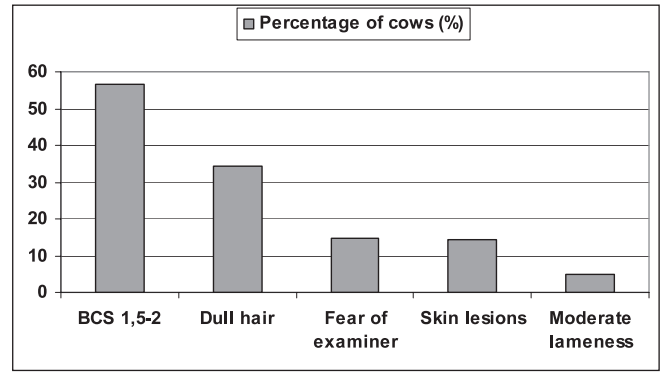

Figure 1: The distribution of the assessed indicators in the 473 dairy cows, except for the body hygiene scores

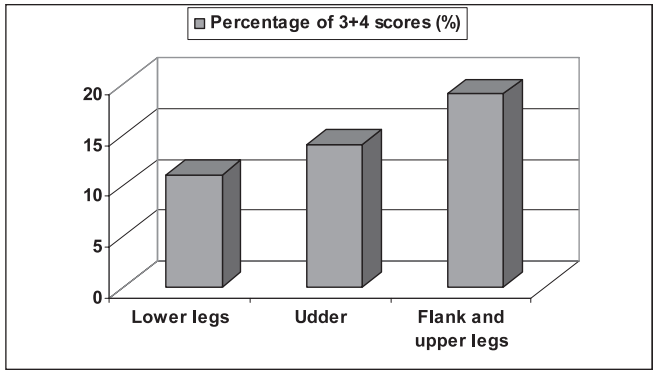

Figure 2: The distribution of 3 and 4 hygiene scores in different body zones of the assessed dairy cows

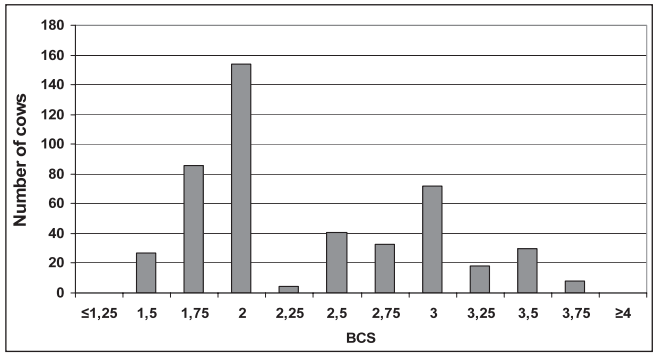

Figure 3: Distribution of body condition scoring in 473 assessed dairy cows

Discussion

In our study the greatest and the most frequent deviation from normality was in BCS. This can be due to the insufficient forage in that period of the year or the inappropriate quality of the feed. Frequently, thin cows do not clinically show the oestrus or become temporally infertile until they begin to gain or at least maintain their body weight. The nutrition of these animals should provide energy for maintenance of their productions and, in the same time, to supply the gain in body weight (9, 10). The body-condition scoring is an assessment tool in the evaluation of the fattening or the body weight loss in dairy cows according to a scale ranging from 1 to 5. In our study the system assigning BCS was the one modified by Thomsen, because it was considered that only extreme deviations from the ideal body condition are relevant from the point of view of the dairy cows' health and welfare and because the original system doubles the time needed for assessment (11). The body condition influences productivity, reproduction, health and longevity of dairy cows. The fat or thin state of cows can constitute an indicator of nutritional, metabolic disorders, health problems or can indicate poor management at farm level (7).

The next indicator modified relative to normality was the fur condition. The aim of this parameter's assessment was to appreciate if the cow is able or not to maintain her skin clean (2, 11). The absence of self-grooming can indicate illness, poor general condition, inability to perform certain movements. Our study's result could be influenced by the length of the chains that tether the cows, limiting the movement possibilities of the animals.

In the majority of investigated farms the animal – human relationship could be considered as good, taking into account that only 69 of the assessed 473 cows showed fear at the approach of the examiner. The measurement of the flight distance is a recognised method in the evaluation of an animal's reaction to humans (15,16). The test can be influenced by different factors (lameness, the social environment of the testing, the past experiences of the animal, the observer known/unknown for the animal etc). It generally reflects the quality and quantity of the animals' manipulation by man and the human-animal relationship (stockmanship). If the animal allows the participant to the test to come within a small distance, this means that the animal does not show fear. In this case, all daily inspections and manipulation procedures will be less stressful for the animal. Thus, it is a good indicator of „positive health" (13). It was stated that the nature of the animal-stockman relationship influences the behavior, milk production and welfare of dairy cows (17). Hemsworth et al. (15) established a correlation between the cattleperson's attitude and the dairy cow's fear and milk production, indicating a possible way to decrease the fear and increase the productivity through revision of the stockperson's attitude and behavior. In extensive breeding systems for dairy cows stockmanship is better, comparative to intensive breeding (8) because of the smaller number of animals and due to their frequent contact with humans at feeding, watering, milking, cleaning the shelter, all this processes being done by manpower.

The skin lesions had a low percentage in our study, comparative to the results of other researches (18), probably due to the simple barns, with only a few equipments, and due to the straw bedding used. Skin lesions (hair loss, small wounds) were possible, especially in the neck region, caused by the chain used for the cows' tethering. The cows (14 cows) presenting lesions (hair loss, swelling of the skin, small wounds) on their legs had the locomotion score of 3 (moderately lame). These lesions not only cause

pain, but can also indicate problems of welfare and production. The skin lesions and swellings reflect the impact of the nearest environment on the animals' bodies (13). Injuries can be caused through the animals' contact with hard floors, with the confinement system, with the watering and feeding eaves or by hiting other hard elements inside the shelter.

Regarding lameness, it had a reduced proportion (Figure 1). This result is surprisingly low compared with the results of other studies. Thus, several authors showed that lack of exercise and pasture lead to increasing feet-problems (3, 19). However, recent figures for the prevalence of lameness in European countries range from 22% (7) to 45% (20) for loose-housing systems and from almost 1% to 21% for systems in which cows are tied for at least part of the time (21). Lameness evaluation in cattle and the evaluation of gait abnormalities are subjective. The locomotion assessment system suggested by Sprecher and others (1997) was used because it presents clear and objective descriptions which differentiate between scores. Lameness represents a major welfare problem in dairy cows, inducing pain and long-term discomfort.

The body hygiene was evaluated through the percentage of the cows with the 3 and 4 scores in three body regions: lower and upper legs, flank and udder. A high percentage of 3 and 4 scores indicate a poor, unacceptable hygiene, with severe consequences on the cows' health, production and welfare. The obtained results indicate lower percentages of the 3 and 4 scores in the three body regions, comparative with the results of other studies (12, 21, 22). Also, one should note the low percentage of the dirty lower legs in comparison with the area of upper leg and flank, similar to the specifications in the scientific literature. It is asserted that cows housed in tie-stall shelters have more elevated hygiene scores in the body region of the upper leg and flank than in the area of lower leg, due to the decubital resting in the manure deposits in the stalls (12). This body region can also become dirty in poorly maintained stalls, with manure on the separating elements or through the movements of the dirty tail around the hind quarter of the body (21). The body hygiene assessment can provide more information about the animal's comfort and attitude of the stockperson and his attention for the animal.

The obtained results indicate that more than a half of the assessed cows were thin, which has negative impact on their health and welfare. The main causes affecting the dairy cows' welfare in small farms in the North-East of Transylvania are inappropriate feeding and the tied housing system.

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PRESOJA DOBREGA POČUTJA KRAV MOLZNIC V VEZANI REJI NA 52 MANJŠIH KMETIJAH V SEVERNOVZHODNI TRANSILVANIJI (ROMUNIJA) NA PODLAGI OCENJENIH ZUNANJIH LASTNOSTI ŽIVALI

S. Popescu, C. Borda, C. D. Sandru, R. Stefan, E. Lazar

Povzetek: Cilj dela je bil oceniti dobro počutje krav molznic na manjših kmetijah v severovzhodni regiji Transilvanije v Romuniji, na podlagi več parametrov, povezanih z živalmi: ocenjevanje telesne kondicije (BSC), telesne higiene (čistoča), šepanja, poškodb kože, kakovosti kožuha in razdalje pobega. Ocenjenih je bilo 473 krav molznic, nastanjenih v vezani reji na 52 manjših kmetijah (5-20 glav goved na kmetijo). S specifičnimi metodami so bili določeni kazalni parametri. Med 473 ovrednotenimi kravami molznicami je 267 krav (56,45 odstotka) imelo BSC med 1,5 in 2, kar pomeni, da so bile presuhe, 162 krav (34,25 odstotka) je na hrbtu imelo oslabljeno dlako, 68 (14,38 odstotka) je na različnih delih telesa imelo poškodbe kože, 69 (14,95 odstotka) je ob pristopu opazovalca pokazalo strah in 23 (4,86 odstotka) je bilo zmerno šepavih. Rezultati 3 in 4 glede čistoče telesa so bili pri spodnjih delih okončin 11-odstotni, 14-odstotni v območju vimena in 19-odstotni v območju bokov in zgornjih delov okončin. Pokazalo se je, da je bila več kot polovica ocenjenih krav molznic presuha, kar ima negativen vpliv na njihovo zdravje in dobro počutje. Na dobro počutje krav molznic v severovzhodni Transilvaniji najbolj negativno vpliva neprimerno krmljenje in privezni sistem reje.

Ključne besede: telesna kondicija; telesna čistoča; šepanje; poškodbe kože

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Original Scientific Article

DETECTION AND MOLECULAR CHARACTERIZATION OF A PIGEON VARIANT OF AVIAN PARAMYXOVIRUS TYPE 1 VIRUS (PPMV-1) FROM A BLACKBIRD *(TURDUS MERULA)*

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Summary: Cloacal and tracheal swabs taken from a dead blackbird *(Turdus merula)* were investigated as part of increased virological monitoring of wild birds during 2006, for the purpose of early detection of highly pathogenic avian influenza H5N1 virus. A pigeon variant of avian paramyxovirus type 1 virus (PPMV-1) was isolated by inoculation of the cloacal swab into the allantoic cavity of 10-day-old embryonated specific-pathogen-free chicken eggs. Molecular characterization of the isolate was performed by reverse transcription PCR and sequencing of the partial fusion (F) protein gene, including the region encoding the cleavage activation site of F protein. The PPMV-1 virus isolated from the blackbird shared the highest partial F gene nucleotide and amino acid sequence identity with PPMV-1 viruses isolated from the free-living and domestic pigeons in Slovenia between 2004 and 2006. The PPMV-1 strain isolated from the blackbird was classified, together with other PPMV-1 viruses associated with the ongoing panzootic in pigeons, into linage 4 sublineage 4b, group 4bii. The PPMV-1 virus isolated from the blackbird has a ^{112}RRQKRF^{117} sequence, associated with virulent avian paramyxovirus type 1 viruses (Newcastle disease viruses), at the F protein cleavage site. To the best of our knowledge this is the first description of the detection of a PPMV-1 virus in a blackbird.

Key words: blackbird (*Turdus merula*); avian paramyxovirus type 1; NDV; F gene sequences; phylogenetic analysis

Introduction

Avian paramyxovirus type 1 virus (APMV-1) of pigeons (PPMV-1) is an antigenic variant of Newcastle disease virus (NDV) of chickens, which causes an autonomous Newcastle disease (ND)-like infectious disease of pigeons (1). The APMV-1 viruses, including PPMV-1, are members of the genus *Avulavirus* (2) within the family *Paramyxoviridae* (3).

ND, included in the Office International des Epizooties (OIE) listed diseases, is a highly contagious and devastating avian disease and, in spite of control measures and vaccination, often manifests itself in epizootics (4). Clinical signs may vary from extremely mild respiratory or enteric diseases (avirulent viruses) to severe systemic infection resulting in high mortality (virulent viruses) and characterized by very rapid spread (5).

The amino acid motif at the precursor fusion protein (F_0) cleavage site has the major influence on the pathogenicity of APMV-1 viruses (6, 7). Different pathotypes of APMV-1 viruses are characterized by their amino acid sequence around the cleavage-site of F_0 (8), which require post-translational cleavage by the host proteases to produce disulphide-linked active F_1 and F_2 subunits of the F protein (9).

Molecular characterizations of APMV-1 strains have mainly considered the F and M genes. The most studied molecular pattern has been the F protein cleavage site (10, 11, 12).

As one of the major antigenic determinants of NDV, the F protein is likely to display greater genetic variation than internal genes. This characteristic is important for studying fairly closely related populations, in which a more conserved gene may show in-

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sufficient sequence variation to allow evolutionary theories to be inferred (13).

Nine NDV genotypes have been described for epizootic NDV strains worldwide (14, 15, 16). Most PPMV-1 strains have been classified into subgroup VIb within genotype VI of NDV by phylogenetic analysis of the region, including the F protein cleavage site (1, 17).

Nine NDV genotypes have recently been reclassified into six broadly distinct lineages (1 to 6) (5). PPMV-1 viruses associated with the ongoing panzootic in pigeons have been placed into sublineage 4b of lineage 4 (13).

Groups or lineages created by phylogenetic analysis appear to be largely congruent and reflect the degree of genetic diversity of NDV and epidemiological associations, such as geographical and/or temporal restrictions of the viruses, the origin of the viruses and the spread of the outbreaks (1).

PPMV-1 strains caused outbreaks among racing and show pigeons in Europe in 1981 and re-emerged in 1985, causing a panzootic that continues to this day (1, 13, 18, 19, 20, 21, 22,). The origins of the panzootic in pigeons appeared to be in the Middle East during the late 1970s and the evidence for this was the fortuitous isolation of PPMV-1 virus in Iraq in 1978 (23). Clinical signs of the infection of pigeons generally include a series of nervous disorders: paralysis, torticollis and watery green diarrhea (24).

Most PPMV-1 strains have had reduced virulence for chickens (25, 26, 27, 28) but, in the most cases, PPMV-1 isolates have increased their virulence for chickens after passages (25, 29, 30).

In addition to pigeons, doves and chickens, PPMV-1 viruses have also been isolated from kestrels, falcons, cockatoos, budgerigars, pheasants, swans and grey partridges (19, 20, 31, 27, 13, 32). Isolation of PPMV-1 virus from a passerine bird (Passeriformes), a robin (*Erithacus rubecula*), has recently been reported (33). Passerine birds are not generally considered important in the epizootiology of APMV-1 viruses (34). Nevertheless, recently published data have shown that passerine birds (an order with over 5000 individual species) can sporadically be infected with APMV-1 viruses (35).

Materials and methods

Isolation of the virus

Cloacal and tracheal swabs taken from a dead blackbird (*Turdus merula*) were submitted to the Institute of Poultry Health, Veterinary Faculty, University of Ljubljana as a part of increased virological monitoring of wild birds during 2006, for the purpose of early detection of the highly pathogenic avian influenza (HPAI) H5N1 virus.

Isolation of the virus was performed by the inoculation of cloacal and tracheal swabs, obtained from the dead blackbird, into the allantoic cavity of 9-to-10-day old embryonated specific-pathogen-free chicken eggs (Lohman, Cuxhaven, Germany) as described by Alexander (36). Briefly, 2ml of sterile phosphate-buffered saline, including antibiotics, 5,000IU/ml of penicillin, 5,000µg/ml of streptomycin and antimycotics, 12.5µg/ml of amphotericin B (Gibco, Paisey, UK) was added to the dry cloacal and tracheal swab. Swabs were vortexed and centrifuged at 1000 x *g* for 10 min. Next, 0.2 ml of supernatant was inoculated into the allantoic cavity of 9-to-10 day-old embryonating chicken eggs. The inoculated eggs were maintained at 37°C and candled daily. We chilled eggs with dead embryos (as they arose) and all eggs were chilled 5 days post inoculation. Allantoic fluids were collected and tested for hemagglutination activity. Isolates were identified through a haemagglutination inhibition test by using APMV-specific antiserums (APMV-1 to APMV-9, excluding APMV-5) and AIV-specific antiserums (H1N to H16N) (36) provided by Veterinary Laboratory Agency, Weybridge, UK. Infective allantoic fluids were stored at -70°C.

RNA extraction, reverse transcription, PCR, and nucleotide sequencing

RNA was extracted from infectious allantoic fluid by QIAamp Viral RNA Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

A region of approximately 454-bp of the F gene of APMV-1, including the F protein cleavage site, was amplified by the oligonucleotide primer pair: F-OPU: 5'-TTG A**Y**G GCA G**R**C CTC TTG C-3' and F-OPL: 5'-TGC ATC TTC CCA ACT GCC ACT-3' (37), with two modifications of the primer F-OPU indicated in bold.

OneStep reverse transcriptase (RT)-PCR Kit (Qiagen, USA) was used for genomic RNA amplification. RT-PCR was performed by uninterrupted thermal cycling with the following program: 30 min at 50°C for RT; RT inactivation at 95°C for 15 min was followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min.

The amplified products were analyzed by electrophoresis on a 1.8% agarose gel stained with the ethidium bromide. The DNA fragments were purified with a Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). Direct double-stranded nucleotide sequencing was completed by an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Woolston, UK) and oligonucleotide primers used for RT-PCR. Reactions were analyzed with an ABI3730xl Genetic Analyzer (Applied Biosystems).

Sequence analysis

The genetic relationship between the partial nucleotide sequences of the F gene of APMV-1 isolated from the blackbird, SLO/12/06 and respective gene sequences of APMV-1 isolates published in GenBank was evaluated.

MEGA 3.1 software (38) was used for editing the nucleotide sequences and deducing the amino acid sequences. Nucleotide and deduced amino acid sequences were aligned with ClustalW software (39). Phylogenetic analyses were constructed with MEGA 3.1 software using the neighbor-joining method with the Kimura-2 parameter substitution model and 1000 bootstrap replicates to assign a confidence level to branches.

Sequences used for pairwise comparisons on F gene

PITTDO0177 (AY175759); PDECT95200 (AY471772); PDECT95204 (AY175752); PTRBU95211 (AY471773); PUKBR84261 (AY471853); PITDO00289 (AY471846); PITPH95294 (AY471839); PITDO01321 (AY471742); PITTD96334 (AY471843); PAEKE99364 (AY471785); PAEKE98373 (AY471783); PILSW01382 (AY471787); PAEKE98398 (AY471784); PUKDO96438 (AY471817); PUKPH96441 (AY471818); ACAGL90270 (AY135756); Q-GB506/97 (AF109887); FIVi1001/96/1 (AF091623); Q-GB445/97 (AF109886); HFRDK77188 (AY135758); PUKPI88224 (AY471830); PUKPI93396 (AY471816); PUKPI89229 (AY471826); PUKPI91233 (AY471800); PUKPI90236 (AY471798); PITTD96334 (AY471843); PITPI96407 (AY471841); PITPH95294 (AY471839); SLO 349/01 (DQ007545); SLO 912/08 (GU002444); SLO 17/04 (GU002428); SLO 75/06 (GU002442); SLO 218/05 (GU002434); SLO 802/05 (GU002438); SLO 718/05 (GU002436); SLO 872/04 (GU002432) and SLO 263/04 (GU002430).

The accession number of the strain SLO/12/06 is GU002440.

Results

Virus isolation

A haemagglutinating agent was isolated by inoculation of the cloacal swab taken from a dead blackbird (*Turdus merula*) into the allantoic cavity of 10-day-old embryonated SPF chicken eggs. The isolate was identified as the APMV-1 virus through a haemagglutination inhibition test by using APMV specific antiserums (36), provided by the Veterinary Laboratory Agency, Weybridge, UK. The presence of avian influenza viruses (AIV) was excluded through a haemagglutination inhibition test.

Molecular characterization of isolate

RNA was extracted from infectious allantoic fluid and a region of approximately 454-bp was amplified by the RT-PCR specific for the F gene region of APMV-1 viruses.

Comparison of the obtained nucleotide and deuced amino acid sequences of the APMV-1 virus isolated from a blackbird in 2006, SLO/12/06, showed that the isolate shared the highest partial F gene nucleotide and amino acid identities with the PPMV-1 strains isolated from the free-living and domestic pigeons in Slovenia between 2004 and 2006. The nucleotide and amino acid identities between the strain SLO/12/06 and the strains SLO/17/04, SLO/872/04, SLO/218/05, SLO/718/05, SLO/802/05 and SLO/75/06 were 99.6% and 98.8 to 100%, respectively.

PPMV-1 isolated from the blackbird has a ^{112}RRQKRF^{117} sequence at the F protein cleavage site (Figure 2).

The strain SLO/12/06 isolated from a blackbird was classified, together with the other PPMV-1 viruses associated with the ongoing panzootic in pigeons, into sublineage 4b of lineage 4 (Figure 1). Strain SLO/12/06 clusters together with PPMV-1 strains isolated from free-living and domestic pigeons in Slovenia between 2001 and 2008, as well as with the European PPMV-1 isolated from pigeons and doves between 1999 and 200l. These strains, together with PPMV-1 strains isolated from other bird species (budgerigar, dove, swan, kestrel and cockatoos), belong to group 4bii of sublineage 4b (Figure 1).

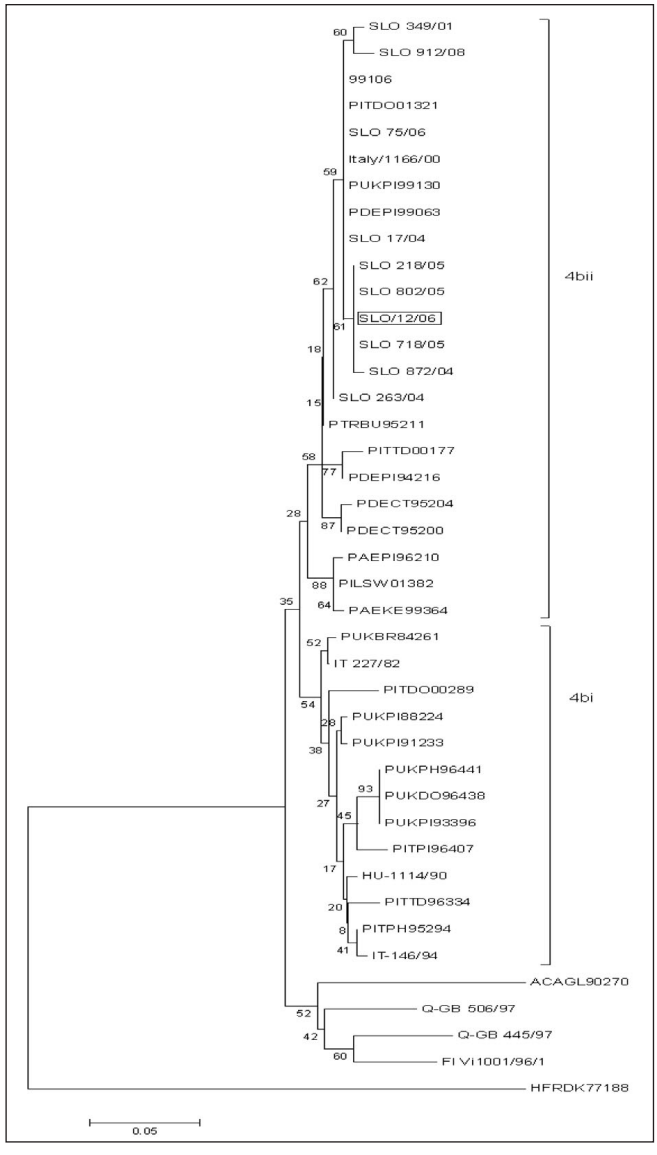

Figure 1: Phylogenetic tree of the region at the 3' end of the fusion protein gene of PPMV-1 viruses isolated from pigeons and other bird species. PPMV-1 strain isolated from the blackbird (framed) cluster together with the strains from group-4bii of sublineage 4b. Phylogenetic analyses were constructed by the neighbor-joining method with the Kimura-2 parameter substitution model and 1000 bootstrap replicates to assign a confidence level to branches. The bootstrap test confidence levels are shown next to the branches

Discussion

An APMV-1 virus (SLO/12/06) was isolated from the cloacal swab of a dead blackbird during the regular monitoring of wild birds for the purpose of early detection of the highly pathogenic avian influenza (HPAI) H5N1 virus. On the basis of the results of molecular characterization of the partial F gene, we can conclude that APMV-1 virus isolated from the blackbird belongs to the pigeon variant of avian paramyxovirus type 1 (PPMV-1) (Figure 1). The strain SLO/12/06 isolated from the blackbird was classified, together with other PPMV-1 viruses associated with the ongoing panzootic in pigeons, into group 4bii sublineage 4b (Figure 1). Group 4bii includes PPMV-1 isolated from pigeons and other bird species from Europe, North America and Asia. Isolates from the group 4bii have been the predominant strains in the latter period of the ongoing panzootic of PPMV-1 viruses in pigeons, with the number of the isolations of viruses from group 4bi diminishing from the late 1980s onwards (13).

The finding that the recently isolated strain from the blackbird, SLO/12/06, is highly related to the strains obtained from domestic pigeons (SLO/17/04 and SLO/872/04) and strains from free-living pigeons (SLO/218/05, SLO/718/05, SLO/802/05 and SLO/75/06) (Figure 1) suggests that the same strains have been circulating between the population of free-living and domestic pigeons and that the blackbird was probably infected from one of them.

PPMV-1 isolated from the blackbird has the ^{112}RRQKRF^{117} sequence at the F protein cleavage site. This sequence, found in the majority of recently isolated PPMV-1 viruses (4, 28), is identical to that associated with highly virulent ND viruses (8). However, this prerequisite for high virulence has not always correlated with the pathogenicity of PPMV-1 strains for chickens (30, 40), although it has been demonstrated that the virulence of the majority of PPMV-1 viruses for chickens is greatly increased following three to four passages through this host (25). The PPMV-1 virus isolated from the blackbird has amino acid sequences associated with virulence at the fusion protein cleavage site and, as such, has to be considered a potential threat for poultry.

To the best of our knowledge, this is the first description of the detection of PPMV-1 virus in a blackbird. This indicates that PPMV-1 viruses may infect this bird species. However, we can not draw any conclusion about the virulence of the PPMV-1 virus and the disease that the virus may cause in these birds, because no post mortem examinations of the dead blackbird were performed.

The incidence of APMV-1 viruses in wild passerine birds was investigated over a three-year period. The results of the study showed that cloacal swabs taken from 598 passerine birds, including 11 blackbirds, between 2004 and 2006, were negative for APMV-1 viruses by virus isolation on embryonated

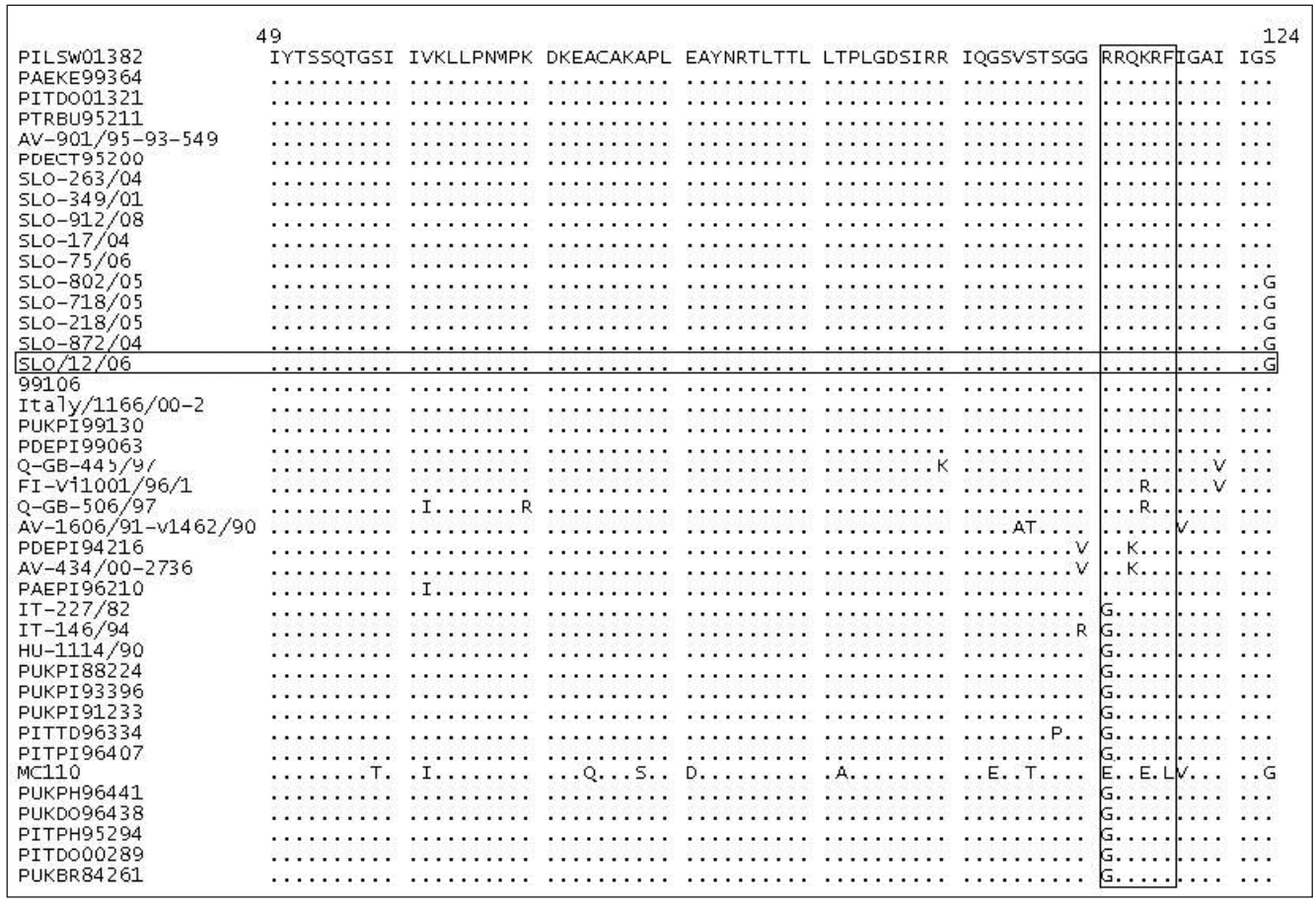

Figure 2: Amino acid sequence alignment of partial PPMV-1 viruses' fusion protein sequences of PPMV-1 isolated from the blackbird and other PPMV-1 strains used in the study. The fusion protein cleavage site sequence from position 112 to 117, considered as a major determinant of strain pathogenicity for poultry, and partial F protein amino acid sequence of PPMV-1 virus isolated from a black bird are boxed

SPF fowl eggs (41). These results, as well as other data from the literature indicate that song birds play only a minor role as a potential disseminator of APMV-1 viruses (35).

Nevertheless, PPMV-1 viruses, which are endemic in the pigeon population in many European countries, can infect robins (33) and blackbirds as reported in the present study. The spread of the virus in the population of passerine birds and the threat that this could represent to these bird species and to poultry production should therefore further investigated.

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IZOLACIJA IN MOLEKULARNA KARAKTERIZACIJA GOLOBJE VARIANTE AVIARNEGA PARAMIKSOVIRUSA TIPA 1 (PPMV-1), UGOTOVLJENEGA PRI KOSU *(TURDUS MERULA)*

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Povzetek: Brisa sapnika in kloake, odvzeta mrtvemu kosu smo preiskali v sklopu povečanega virološkega nadzora prostoživečih ptic, da bi dovolj zgodaj ugotavljali zelo patogen virus aviarne influence H5N1. Iz brisa kloake smo pri 10 dni starih kokošjih embriih brez specifičnih protiteles izolirali golobjo različico aviarnega paramiksovirusa tipa 1 (PPMV-1). Molekularno karakterizacijo izoliranega virusa smo izvedli z metodo obratnega prepisa in verižne reakcije s polimerazo ter določanjem zaporedja nukleotidov na območju gena za fuzijski (F) virusni protein, vključno z območjem, ki določa mesto cepitve virusnega proteina F. Zaporedji nukleotidov in aminokislin območja na genu za protein F pri virusu PPMV-1, ugotovljenem pri kosu, sta bili najbolj podobni zaporedjem nukleotidov in aminokislin pri virusih PPMV-1, ugotovljenih pri prosto živečih in domačih golobih med leti 2004 in 2006 v Sloveniji. S filogenetsko analizo smo virus uvrstili v linijo 4, podlinijo 4b virusov PPMV-1. Zaporedje aminokislin na mestu cepitve proteina F pri virusu PPMV-1, ugotovljenem pri kosu, je bilo ^{112}RRQKRF^{117}. Ugotovljeno zaporedje aminokislin je značilno za virulentne aviarne paramiksoviruse tipa 1 (viruse newcastelske bolezni). Glede na nam dosegljive podatke menimo, da gre za prvi opis izolacije in molekularne karakterizacije virusa PPMV-1, ugotovljenega pri kosu.

Ključne besede: kos *(Turdus merula)*; aviarni paramyxovirus tipa 1; NDV; zaporedja nukleotidov in aminokislin gena za protein F; filogenetska analiza

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Original Scientific Article

THE PRESENCE OF SMOOTH MUSCLE CELLS AND ELASTIC FIBERS IN THE BULL VESICULAR GLAND

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Summary: Vesicular gland is a compact, lobulated organ surrounded by a capsule of dense irregular connective tissue with a few smooth muscle cells. Smooth muscle cells and elastic fibers as supporting and contractile structures were examined in the vesicular glands (VG) of the bull by the immunohistochemical method. The elastin and -smooth muscle actin (α-SMA) antibodies were used for their detection. Bundles of smooth muscle cells (SMC) positive for smooth muscle actin form thick muscular layer of the gland. Elastic fibers as loose network are inserted among the muscle cells and the bundles of the muscular layer. Thick connective tissue trabeculae rich in smooth muscle cells were seen to arrise from the muscle layer and enter the mucosa. In the stroma of the mucosa layer, smooth muscle cells form bands of different dimensions and arangement. Individual smooth muscle cells were seen just beneath the secretory epithelium. An accumulation of elastic fibers was seen in the connective tissue that separates the secretory alveoles. Dense concentration of elastic fibers have been seen to be located close to secretory epithelium. High accumulation of elastic fibers of the vesicular gland together with smooth muscle cells are supposed to participate in a rapid releasing of the secretory product and rearrangement of the mucosa during and after the ejection.

Key words: vesicular glands; smooth muscle cells; elastic fibers; bull; immunohistochemistry

Introduction

The vesicular gland (*glandula vesicularis*), seminal vesicle are androgen-dependent secretory glands of the male genital tract which together with prostate produce a bulk of the seminal secretions. The vesicular gland was studied in relation to stromal maturation during the ontogeny and in the adult stromal composition after hormonal influences or in tumours. Stromal development of the vesicular gland of the rat was examined by immunocytochemical methods during the pre- and postnatal developmental periods (1). Histological quantitative analysis of collagen and smooth muscle in seminal vesicle stroma after estradiol-17β administration to the immature castrated rat were performed (2).

Only a few studies are there relating the presence of contractile smooth muscle cells (SMC) in the vesicular gland. The distribution of myofibroblasts (MFb) in the stroma of a normal vesicular gland was studied (3). An electron microscope examination revealed the presence of spindle or stellate cells classified as myofibroblasts, distributed in the stroma of the lamina propria. These cells were found to be major stromal components in renal pelvic and ureteral, and in cancers of different organs (4-8). An immunoelectron microscopic examination showed that cells beneath the seminal vesicle epithelium were positive for smooth muscle actin (3).

The reports relating the presence of elastic fibers in the male accessory glands in animals are scarce and were documented mostly in organs of the urinary tract. Distribution of elastic fibers in the upper urinary tract of the human fetus have been published (10). An abundance of elastic fibers among the smooth muscle bundles the human male urethra was (9). In the animals, investigation of the elastic fibre system of the female canine urethra was per-

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formed (11). Morphometric studies on elastic fibers in the urethra have been made in the dog (12), bitch (13), and cat (14). Both male and female guinea pigs showed great amounts of circularly disposed elastic fibers in the vesicourethral junction (15). This particular disposition of fibers may be responsible for imparting resiliency and plasticity to the vesicourethral junction. Elastic fibers in this place have been assumed to contribute to the resting urethral closure pressure. In the bladder base, vesicourethral junction and urethra, the elastic fibers may be partly responsible for the passive occlusive force in this region. The presence of elastic fibers in the vesicular glands, to our knowledge, was not documented. The aim of this work was to study immunohistochemically the distribution of elastic fibers and contractile smooth muscle cells in the bull vesicular glands.

Material and methods

Vesicular gland from five adult bulls was used in this study. The samples of vesicular gland were taken out at the local slaughterhouse immediately upon death. Samples of the tissue were fixed in 10% formaldehyde in 0.2 mol phosphate - buffered saline (PBS) for 24h and routinely embedded in paraffin. The sections 5-μm-thick were cut and stained with haematoxylin-eosin as a general stain. Consecutive sections were used for histological, control and immunohistochemical procedures. For immunohistochemistry, the sections were mounted on slides coated with 3-aminopropyltriethoxysilane.

For immunostaining procedure, histological sections were deparaffinised and rehydrated, pretreated with 3% H_2O_2 in methanol to block endogenous peroxidase activity and preincubated with 2% goat serum to mask unspecific binding sites. Washed sections were incubated overnight with primary antibody – monoclonal mouse anti-α-SMA (Dako), dilution 1:200, and monoclonal mouse anti-elastin (Sigma), dilution 1:5000. The sections were washed in phosphate-balanced salt solution (PBS) and incubated with biotinylated secondary antibody for 30 min. Washed sections in PBS were incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector, Burlingame, USA). After washing with PBS, peroxidase activity was visualized with diaminobenzidine (DAB) and H_2O_2 in TRIS buffer within 5 min at room temperature. Sections were counterstained with Mayer's hematoxylin. For negative controls, the primary antibody was substituted by PBS.

Results

Anatomically, vesicular gland in bull is a compact, lobulated organ, and histologically it is a paired, compound tubuloalveolar gland. A muscular coat consists of an inner layer of circularly arranged smooth muscle cells and an outer layer in which the smooth muscle cells have a longitudinal orientation. External to the muscle coat is adventitia, consisting of loose connective tissue. The highly vascularised loose connective tissue of the lamina propria – submucosa is continuous with the dense connective tissue trabeculae. The interlobular septa are predominantly muscular, derived from the thick tunica muscularis. The mucosa of the vesicular gland consists of secretory alveolae separated by loose connective tissue trabeculae of different thickness. Intralobular secretory ducts drain the slightly coiled tubular portions of the tubuloalveolar gland. The secretory columnar cells and the basal cells have lipid droplets, often in an infranuclear position. An immunohistologic examination showed that the cells positive for α-smooth muscle actin (α-SMA) were observed to form thick tunica muscularis (Fig. 1). The capsule contains a few smooth muscle cells. Groups of smooth muscle cells were observed to arise from muscle layer and enter the mucosa layer. The trabeculae of the lamina propria of different thickness contain bands of SMC which were seen to be localized centrally. Single smooth muscle cells forming incomplete layer were observed beneath the secretory epithelium (Fig. 2). Numerous small blood vessels rich in SMC were present in mucosa layer.

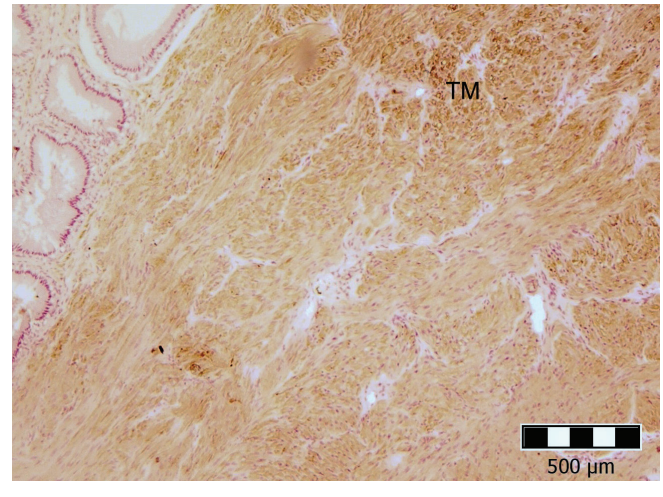

Figure 1: Positive cells for α-smooth actin (brown) forming thick tunica muscularis (TM). Nuclei were counterstained with Meyer's hematoxylin

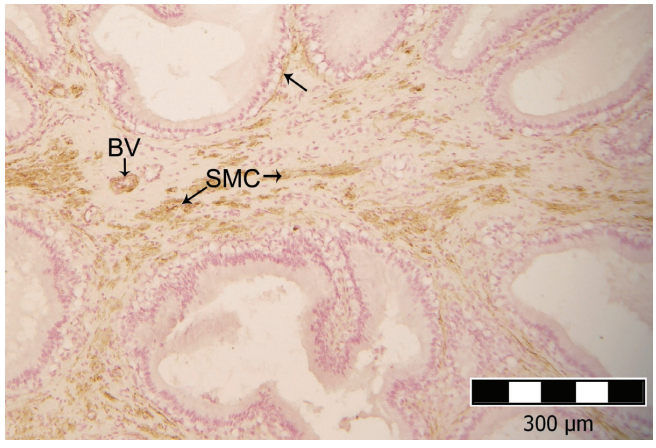

Figure 2: Positive cells for α-smooth actin (brown) localized in trabeculae of lamina propria. Smooth muscle cells are also beneath the secretory epithelium (arrows). Blood vessels (BV) containing smooth muscle cells (SMC). Nuclei were counterstained with Meyer's hematoxylin

Elastic fibers in the vesicular gland were present within all layers – capsule, the muscular layer, submucosa and mucosa. Many elastic fibers occur in the muscular layer and were distributed among bands of the SMC (Fig.3). In the submucosa and in the mucosa layer, elastic fibers were present throughout the area of the lamina propria, forming thick and thin trabeculae. In the connective tissue of mucosa, elastic fibers were seen to be distributed regularly among the muscle cells and collagen fibers. An accumulation of elastic fibers occurred next to secretory alveoles to form thick elastic membranes just beneath the secretory epithelium (Fig.4).

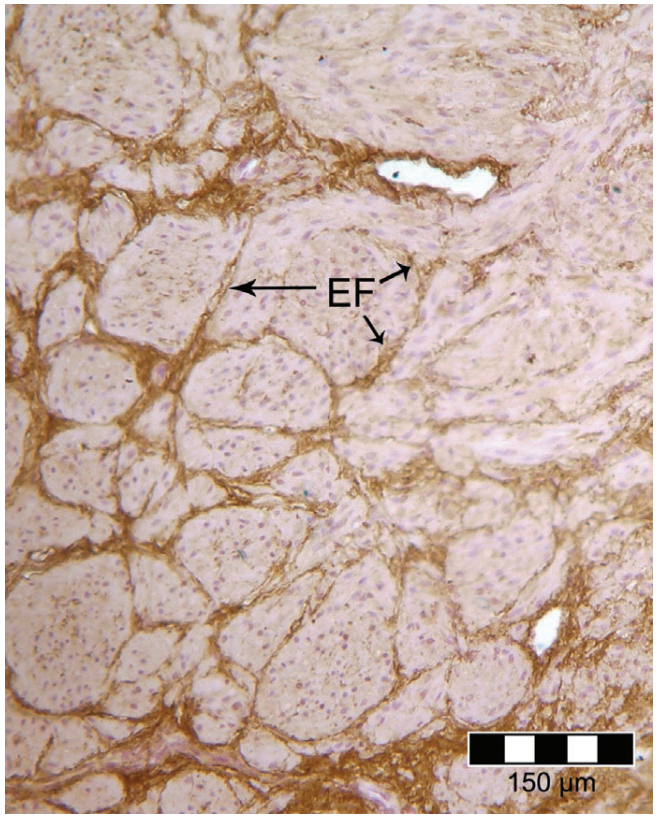

Figure 3: Elastic fibers (EF) are distributed among the bands of the muscular layer. Nuclei were counterstained with Meyer's hematoxylin

Discussion

In the bovine vesicular gland both structures studied - the smooth muscle cells and elastic fibers - were seen to be richly developed. As for cells reacting positively for α-SMA, distributed in all layers, the muscle layer, submucosa and mucosa layer. In the muscle layer, which in bull is very thick, the arrangement of muscle cells is typical for visceral organs. It consists of typical smooth muscle cells which were distributed in bands of different thickness, running mostly circularly. As we have seen, the organization of the SMC in this organ is different from that in other accessory glands. Thick muscle layer and rich saturation of the mucosa layer with smooth muscle cells seem to be specific for these sex accessory organs.

The classification of the muscle cells present in the mucosa, namely those beneath the secretory epithelium, is not clear. Though these cells displayed a strong positive reaction for -SMA and presented

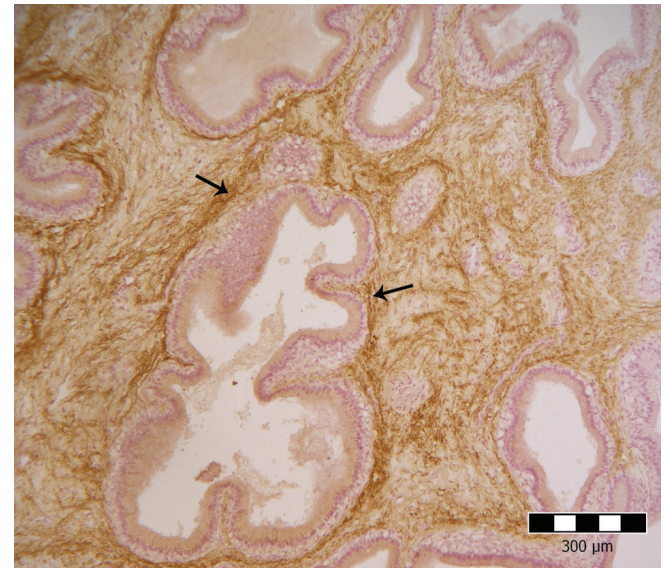

Figure 4: Elastic fibers in the trabeculae of the mucosa form dense notwork. High concentration of elastic fibers are seen beneath the secretory epithelium (arrows). The section was counterstained with Meyer's hematoxylin

spindle shape typical for smooth muscle cells, their cytoplasmic components seemed to be different. The cells lying under the secretory epithelium were seen to be more slender with a small amount of cytoplasm, whereas the cells inside the trabeculae were seen to be rich in cytoplasm. Smooth muscle actin was also proved histochemically in smooth muscle cells of other organs and cells: in pericytes of blood vessels (16), in human normal testicular stroma (17, 18) in normal pancreas and various pancreatic lesions (19), and in highly differentiated fibroblastic cells, the so-called myofibroblasts (20-22). The cells positive for alpha-smooth muscle actin in the stroma of normal seminal vesicles just beneath the epithelium, which the authors characterized as myofibroblasts were observed (3). Though the cells under the epithelium and those in the septa of vesicular gland express α-SMA typical for smooth muscle cells, the specific environment and morphological features of these cells may have also another function than that ascribed for SMC (23).

There are more data about the presence of myofibroblasts in various organs, however, there are still doubts whether these cells are true myofibroblasts (24, 25). Evidences reported (3) that the majority of myoid cells in human testicular seminiferous tubules are myofibroblasts rather than smooth muscle cells, and supposed that these myofibroblasts may play a role in sperm transport. This mechanism has been shown also in the rat seminal vesicle (26). Myofibroblasts have also been identified to play a role in tissue contraction during wound healing and contraction (27-30) and in various pathological conditions and organ fibrosis (31-33). The muscle cells observed in the mucosa layer of vesicular gland may have the functional properties of byofibroblasts.

The connective tissue of the mucosa of the vesicular gland is specific and differs from similar tissue in other glands. The vesicular gland and prostate are androgen-dependent secretory glands of the male genital tract. They produce the bulk of the seminal secretions. In the vesicular gland, androgen receptors were observed in the lamina propria (1). Lamina propria and mainly subepithelial area with muscle cells are very rich in nerve fibers. Around the glandular secretory alveoles and namely below the epithelial lining of the glandular duct, a tightly woven subepithelial plexus was seen which sends short penetrating branches into the basal zone of the epithelium (34).

A dense concentration of the elastic fibers was seen in the bull vesicular glands. Only in a few studies these fibers were observed in animals. Elastic fibers have been demonstrated in the urethra of the dog (12), bitch (13), and cat (14). Both male and female guinea pigs showed great amounts of circularly disposed elastic fibers in the vesicourethral junction (15). It seems that vesicular glands are specific and characteristic with such concentration of elastic fibers.

In conclusion, in the bull vesicular gland smooth muscle cells and elastic fibers form an important structure of the organ. Both structures are localized within the muscle layer, submucosa and mucosa. The organization of muscle cells and elastic fibers is related to the process of the ejection of sperm and seminal plasma. The particular disposition of elastic fibers may be responsible for imparting resiliency and plasticity to the vesicular gland, allowing it to distend and recoil in response to ejaculation.

Acknowledgements

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PRISOTNOST GLADKIH MIŠIČNIH CELIC IN ELASTIČNIH VLAKEN V MEHURNICI BIKOV

E. Marettová, J. Legáth

Mehurnica je čvrst, režnjičast organ, obdan s kapsulo iz gostega, neenotnega vezivnega tkiva, ki vsebuje nekaj gladkih mišičnih celic. S pomočjo imunohistokemičnega barvanja smo proučevali gladke mišične celice in elastična vlakna kot podporne in krčljive strukture v mehurnici bika. Za ugotavljanje so bila uporabljena protitelesa proti elastinu in gladkemu mišičnemu aktinu α (α-SMA). Ugotovljeno je bilo, da snopi gladkih mišičnih celic (SMC), pozitivnih na aktin gladkih mišičnih celic, oblikujejo obilno mišično plast žleze. Elastična vlakna so kot ohlapna mreža vložena med posamezne mišične celice in snope mišičnine. Debelo vezivno tkivo, bogato s trabekulami, v gladkih mišičnih celicah prehaja iz mišične plasti v sluznico. V podpornem tkivu sluznice gladke mišične celice oblikujejo trakove različnih dimenzij, ki so različno razporejeni. Pod epitelijem so bile vidne posamezne mišične celice. Elastična vlaka, ki ločujejo izločevalne mešičke, so bila nakopičena v vezivnem tkivu. V bližini epitelija smo opazili goste snope elastičnih vlaken. Gosti snopi elastičnih vlaken v mehurnici naj bi skupaj z gladkimi mišičnimi celicami sodelovali pri hitrem sproščanju izločkov in prerazporeditvi sluznice pred ejakulacijo in po njej.

Ključne besede: mehurnica; gladke mišične celice; elastična vlakna; bik; imunohistokemija

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Original Scientific Article

VALIDATION OF TWINSENSOR^{BT}, SCREENING TEST FOR THE DETECTION OF ß-LACTAMS AND TETRACYCLINES IN MILK, AND COMPARISON TO DELVOTEST® SP-NT

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Summary: Antimicrobial drugs have been widely used in dairy industry for more than five decades generally to prevent or treat mastitis. The detection of antibacterial residues in milk requires screening methods that are simple, quick and sensitive at antibiotic concentrations close to the maximum residue limit (MRL). A new competitive receptor test Twinsensor^{BT} was validated and compared with Delvotest®SP-NT an agar diffusion microbiological test. Both tests were designed for screening antimicrobial substances in milk. The performance criteria described by the Commission Decision 2002/657/EC, ISO 13969:2003, ISO 18330:2003 and Guide for analytical validation of screening methods (AFSSA Fougères) were used for the validation study. Validation was made on spiked samples of milk with 12 different ß-lactams (penicillin-G, ampicillin, amoxicillin, cloxacillin, nafcillin, cefapirin, cefalonium, cefazolin, cefoperazone, ceftiofur, cefalexin, cefquinome) and 4 tetracyclines (doxycycline, chlortetracycline, oxytetracycline, tetracycline). The Twinsensor^{BT} test was found to be easy to use, with very short incubation period (6 minutes), robust and sensitive to all certified ß-lactame and tetracycline antibiotics at or lower concentrations than EU maximum residue limits, except for nafcillin. The Delvotest®SP-NT on other hand has a longer incubation period (3 hours) and is less sensitive to oxytetracycline, but it can detect a wide range of other antimicrobial substances mostly at or below EU MRLs.

Key words: Twinsensor; Delvotest; milk; antibiotics; screening test; residues; food safety

Introduction

Antimicrobial drugs have been used in dairy industry for more than five decades. They are principally administered to prevent or treat udder infections, but are also applied for the treatment of other diseases. The presence of antimicrobial drug residues in milk is a public health issue (1, 2).

The dairy industry has always been interested in rapid tests to screen the incoming milk on residues of ß-lactam antibiotics in order to prevent technological problems in cheese or yoghurt production (3, 4, 5). To avoid the long incubation period inherent to microbiological inhibitor tests, enzymatic, receptor and immunological tests were developed for a rapid screening of foodstuffs of animal origin on the presence of antimicrobials. The first fast test developed for that aim was the Penzym Test, an enzymatic (carboxypeptidase) colorimetric test, producing a result in 20 minutes. In the late 80s and early 90s, several screening tests with a total test time below 10 minutes (receptor tests SNAP, Charm MRL Beta-lactam Test (ROSA) and βeta s.t.a.r. and immunoassays Lactek and Parallux) became commercially available for monitoring of raw milk on ß-lactams (3). More recently, some rapid tests (Charm MRL-3 and βeta s.t.a.r. 1+1) were adapted to give a test result within 3 minutes, allowing screening of milk at the farm before collection. Also rapid tests for the detection of tetracyclines (SNAP Tetracycline Test Kit, TetraSensor Milk, Charm Tetracyclines - ROSA), sulfamethazine (SNAP Sulfamethazine Test Kit, Charm SMZ), sulfadimethoxine and sulfamethazine (Charm SDSM), gentamicin (SNAP Gentamicin Test Kit), enrofloxacin (Charm ROSA Enrofloxacin) or for a simultaneous detection of ß-lactams and tetracyclines (TwinSensor Milk, Charm ROSA MRLBLTET-3

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and SNAP Duo) are present on the market. Parallux Milk Residue Testing System detects all six major ß-lactams, tetracyclines, spectinomycin, neomycin, streptomycin, spiramycin, sulfa drugs and quinolones in one test in 4 minutes (5).

The ideal screening method would detect most, if not all, antimicrobials at or below their permissible limits or maximum residue limits (MRLs) (1).

Within this paper an evaluation of the performance of the Twinsensor^{BT} (Unisensor Diagnostic Engineering, Belgium) β-lactam-tetracycline test is described and compared to the Delvotest®SP-NT (DSM, Delft, The Netherlands).

Material and methods

Bovine milk free of antimicrobial agents

UHT milk containing 3.5% of milk fat (Prekmurske mlekarne and Ljubljanske mlekarne - different batches) and different samples of raw milk from untreated cows were used. The milk samples were verified by microbiological (plate test acc. to KUNDRAT) and chemical method (LC-MS-MS) as being free of -lactam and tetracycline antibiotics prior to use in the validation study.

Standard solutions and spiked milk samples

Standard solutions and spiked milk samples were prepared in accordance to Commission Decision 2002/657/EC (6), ISO 13969:2003 (7), ISO 18330:2003 (8) and Guide for analytical validation of screening methods (9).

Analyte stock solutions (1 mg mL^{-1}) were prepared from reference standards on a weekly basis in water, methanol, phosphate buffer or DMSO as appropriate and stored at 2-8°C (unless stated otherwise).

Intermediate standard solutions were prepared freshly on a daily basis in distilled water.

Aliquots of the milk were spiked individually with antimicrobial substances on a daily basis. The addition of the spike was set at 0.5 mL of the intermediate standard and adjusted to 50 mL with milk (unless stated otherwise).

Apparatus

Heater Bloc: HeatSensor: 40±3°C

ReadSensor Version 2.1 (77 Elektronika KFT for Unisensor)

Water bath 64±0.5°C

Twinsensor^{BT}

Twinsensor^{BT} test kits were supplied by Unisensor Diagnostic Engineering (Belgium). Twinsensor^{BT} is a competitive test involving two receptors in one single operation. The test requires the use of two elements. The first element is a microwell containing a certain amount of both receptors and antibodies linked to gold particles and the second is a dipstick made of a set of membranes on capture lines. The "control" line printed in red is visible all the time and the other two are specific "test" lines placed on both sides of the control line. The unique line for ß-lactams (penicillins and cephalosporins) is located below the control line while the line relating to tetracyclines is located above it. The assay can either be read visually or instrumentally using the ReadSensor.

The test procedure

The test procedure was carried out exactly as per test kit instructions. 200 µL of the milk sample was applied into the microwell, mixed with the reagents in the microwell and incubated for 3 minutes. The dipstick was dipped into each of the microwell laid in the incubator and incubated for 3 more minutes. Dipsticks were read with a ReadSensor within 15 minutes of performing the test. Dipsticks were visually verified that strip has a valid development: central control line should be visible.

Interpretation of the ReadSensor readings

The visible valid dipstick was inserted into ReadSensor.

A) NEGATIVE readings (Beta: NEG, Tetra: NEG) indicate a negative result — no present ß-lactams or tetracyclines in a milk sample.

B) POSITIVE readings indicate a positive result:

- Beta: POS or LPOS, Tetra: NEG indicate a presence of ß-lactams;
- Beta: NEG, Tetra: POS or LPOS indicate a presence of tetracyclines;
- Beta: POS or LPOS, Tetra: POS or LPOS indicate a presence of ß-lactams and tetracyclines in a milk sample.

Validation experiments

Determination of detection capability CCβ

As screening methods only the procedures that do not exceed 5% of false negative results at concentration of interest can be used.

All 14 antimicrobial compounds specified in the manufacturer's manual and two additional cepha-

losporins used in Slovenia were included in the validation. Because the manufacturer gave a LOD interval (not fixed concentration), that is even variable with the test batch number, we decided to test the concentration at MRL (μgL^{-1}) or just a little bit lower – when possible for every substance. Doxycycline has no MRL set, so it was analyzed at the lowest LOD point specified by the manufacturer. Validation procedure was performed during the period from February 2009 to May 2010. At least twenty different blank milk samples were spiked (unless stated otherwise) and two different Test Kit Batch numbers and two analysts on different days were used for every substance mentioned in the Table 1.

Table 1: Antimicrobial agents included in the validation study

Compound	LOD [a]	MRL in milk [b]	Test conc.[c]
Penicillin-G	2-3	4	4
Ampicillin	3-5	4	4
Amoxicillin	3-5	4	4
Cloxacillin	6-8	30	20
Nafcillin	30-40	30	30
Cefapirin	6-8	60	60
Cefalonium	3-5	20	20
Cefazolin	18-22	50	50
Cefoperazone	3-4	50	50
Ceftiofur	10-15	100	50
Cefalexin	-	100	100
Cefquinome	-	20	20
Doxycycline	20-40	-	30
Chlortetracycline	45-55	100	100
Oxytetracycline	56-75	100	100
Tetracycline	75-100	100	100

[a] Limit of detection as stated in the test instructions Twinsensor^{BT} (μgL^{-1}) (10)
[b] Maximum residue limits: Commission Regulation No 37/2010 (μgL^{-1}) (11)
[c] The concentration we tested (μgL^{-1})

Specificity

Specificity means the ability of the method to discriminate between the tested analyte and other substances, which are chemically related or have a related effect (6).

We tested 12 different ß-lactams and 4 different tetracyclines in different concentrations to test the ability of the Twinsensor^{BT} test to discriminate between them. We also tested milk samples with standard additions of other antibiotics — representative for the antibiotic group according to the Guide for analytical validation of screening methods (9). They should not react positive on this test, because it is commercialized as specific for the screening of ß-lactams and tetracyclines. Compounds we analyzed: streptomycin, gentamicin, erythromycin, tylosin, lincomycin, enrofloxacin, trimethoprim, chloramphenicol and sulfathiazol. Working solutions in milk were made in concentrations: 500 and 250 mgL^{-1}.

Test robustness

Robustness of the method is defined as its susceptibility to minor changes in laboratory conditions (6).

The following robustness parameters were tested: incubation time, incubation temperature, different analysts, different test batch numbers, different bovine milk samples, milk with low pH (spoiled milk) and interfering substances: bronopol (BR).

To test the influence of incubation time we tested 6 different milk samples spiked with penicillin-G (concentration of 4 μgL^{-1}) and 6 blank milk samples. We prolonged first and second incubation period separately from 3 to 6 minutes.

To test the influence of the incubation temperature we changed the heating program on the HeatSensor to only alternative available: 50°C. The optimum temperature of incubation recommended by the manufacturer of the Twinsensor^{BT} Test is 40±3°C. 6 different spiked milk samples (penicillin-G, 4 μgL^{-1}) and 6 blank milk samples were analyzed.

During validation procedure we used 5 different test batch numbers, more than three different packages of the test per batch, changed 3 different analysts, 3 different people preparing spiked samples and the procedure was conducted in longer period of approximately one year.

At least two different test batch numbers were used and two different analysts on two or more different days analyzed altogether 20 or more different spiked milk samples of every substance and concentration validated.

We tested some milk samples with lower pH than normal: 2 different blank milk samples acidified with hydrochloric acid to pH 4.5 and 2 samples naturally spoiled - acidified.

Delvotest®SP-NT

Delvotest®SP-NT test kits were supplied by DSM (Netherlands). The antimicrobial compounds were purchased from Sigma and Aldrich / Fluka / Riedel De Haën Chemicals (Poole, Dorset, UK).

Delvotest®SP-NT is an agar diffusion test based on the inhibition of growth of *Bacillus stearothermophilus*, a thermophilic bacterium highly sensitive to many antimicrobials applied in the dairy industry. The agar contains a standardized number of bacterial spores, select nutrients and the pH indicator bromocresol purple. The assays can either be read visually or by using the AOAC-approved DelvosScan (1, 11).

The test procedure

The test procedure was carried out as per test kit instructions. 100 μL of milk sample was applied into the test ampoule and incubated for 3 hours in the water bath 64±0.5°C.

Interpretation of the results

Results were read visually within 5 minutes of performing the test: yellow colour was interpreted as a negative result, 50% or more intensive purple colour was recorded as a positive test result.

Additional tests

The validation study for Delvotest®SP-NT was already conducted in our laboratory. We made additional tests to validation and used some data from the literature (1, 11).

Results of the validation of the Twinsensor^{BT} and comparison to Delvotest®SP-NT

Determination of detection capability CCβ

We analyzed 69 different samples of antibiotic free (blank) milk and the same ones with a standard addition of antibiotic substances named in the Table 1 (n = 16). The replicates were made on different days, through the period of approximately one year of validation study, with three different analysts and five different batches of the Twinsensor^{BT} test.

Concentrations applied within the validation study were first set at MRL-s (μgL^{-1}) except for the doxycycline (20 μgL^{-1}).

The test showed lower ability to detect the amoxicillin and poor ability to detect nafcillin, doxycycline and cefalexin residues in milk (Figure 1). After the first two applications of doxycycline (20 μgL^{-1}), nafcillin (30 μgL^{-1}) and cefalexin (100 μgL^{-1}) that gave negative result we stopped the validation procedure for those antibiotics. They were additionally tested on different day, with new standard solutions made and different analyst. After two more results for every one of them under these conditions were obtained we analyzed nafcillin and doxycycline in higher concentrations (Table 2). In newer batch numbers of the Twinsensor^{BT} test the LOD for cefalexin was set at more than 750 μgL^{-1} which is much higher than MRL (100 μgL^{-1}) so we decided to stop the validation procedure for this substance.

Doxycycline has no MRL set and LOD of the Twinsensor^{BT} test is around 30 μgL^{-1}.

With exception of nafcillin (LOD is around 60 μgL^{-1}) and cefalexin all the antibiotic compounds listed on the manufacturer's certificate were detected at estimated detection limits and at or below the EU MRLs.

The Twinsensor^{BT} test has a relatively narrow spectrum of antibiotic substances it is sensitive to in comparison to Delvotest®SP-NT (Table 3).

Regarding tetracyclines and ß-lactams used in Slovenia, Twinsensor^{BT} test is more sensitive. Both tests are able to detect those substances at

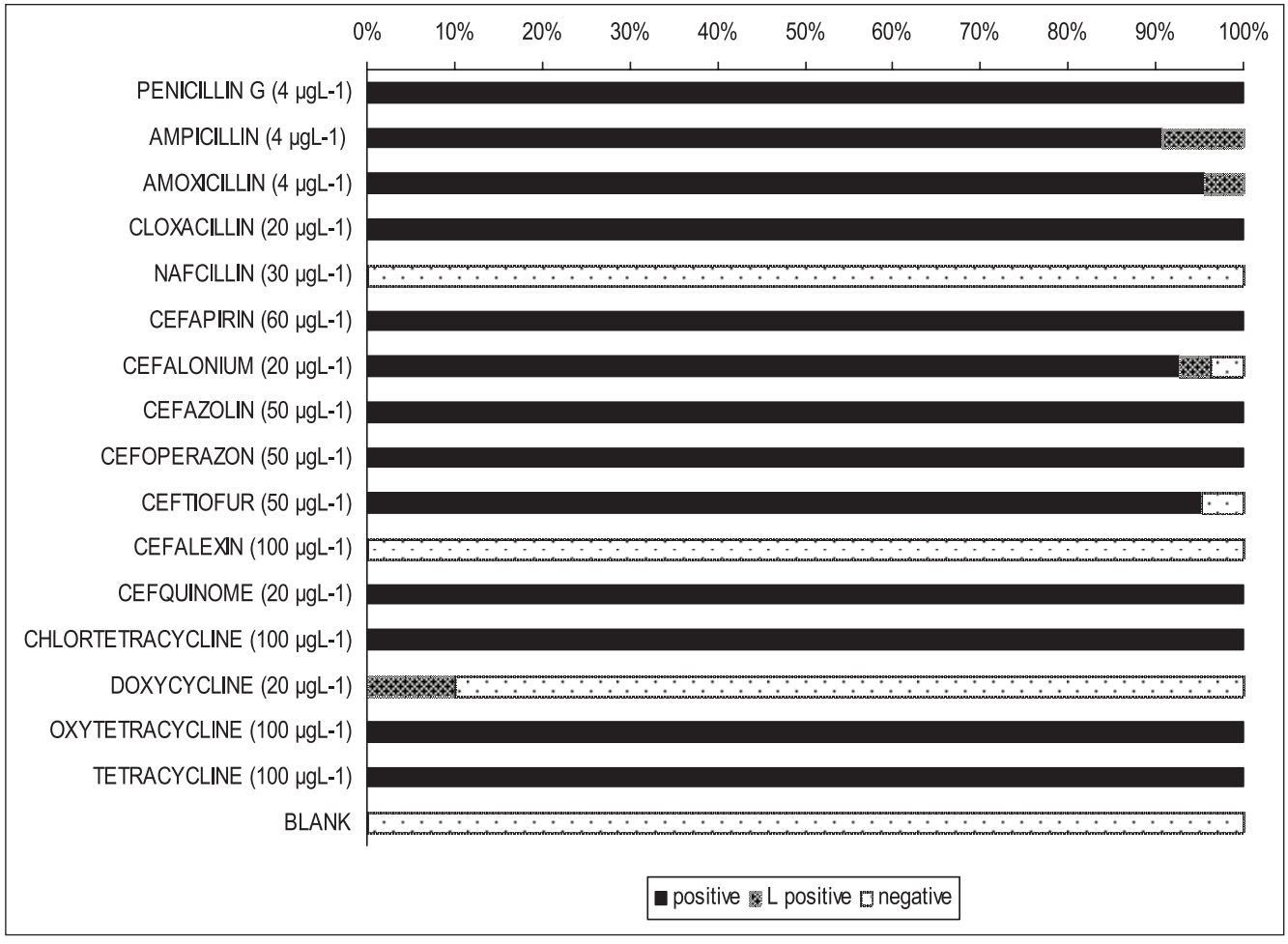

Figure 1: Detection pattern of antimicrobial substances

Table 2: Nafcillin and doxycycline tested in higher concentrations

Compound	LOD [a] (μgL^{-1})	MRL [b] (μgL^{-1})	T. conc. [c] (μgL^{-1})	POS	LPOS	NEG	n
NAFCILLIN	30-40	30	30	-	-	4	4
			40	-	-	4	4
			50	4	1	3	8
			60	20	2	-	22
			80	2	-	-	2
			100	2	-	-	2
DOXYCYCLIN	20-40	-	10	-	-	1	1
			20	-	2	18	20
			30	20	1	1	22
			40	1	-	-	1
			50	1	-	-	1

[a] Limit of detection as stated in the test instructions (10)
[b] Maximum residue limits (MRL) (11)
[c] The concentration we tested (µgL-1)

POS – Number of positive results
NEG – Number of negative results
LPOS – Number of low positive results
n – Number of all tested spiked milk samples

Table 3: Sensitiveness and capability of achieving MRL in milk by Twinsensor^{BT} and Delvotest®SP-NT regarding results of validation and information from the literature - of antibiotic substances allowed for use in Veterinary medicine in Slovenia to treat lactating cows (14)

Antibiotic group	For i/mam application	Other applications	MRL in milk (μgL^{-1})	LOD Delvo (μgL^{-1})*	LOD Twin (μgL^{-1})**
PENICILLINS	Ampicillin	Ampicillin	4	4	3-5
	Amoxicillin	Amoxicillin	4	4	3-5
	Cloxacillin		30	20	6-8
	Penicillin-G	Penicillin-G	4	2	2-3
CEPHALOSPORINS	Cefacetril		125	20	30-40
	Cefalexin	Cefalexin	100	100	> 750
	Cefalonium		20	5-10	3-5
	Cefapirin	Cefapirin	60	10	6-8
	Cefquinome	Cefquinome	20		20-30
		Ceftiofur	100	100	10-15
	Cefoperazone		50	40	3-4
AMINO-GLYCOSIDES	Dihydro-streptomycin	Dihydro-streptomycin	200	300-500	NS
		Gentamycin	100	100	NS
	Kanamycin		150	2500	NS
	Neomycin	Neomycin	500	250	NS
	Streptomycin		200		LS
TETRACYCLINES		Oxytetracyclin	100	250	60-80
	Tetracycline		100	100	80-100
QUINOLONES		Danofloxacin	30		NS
		Enrofloxacin	100		NS
		Marbofloxacin	75		NS
SULFONAMIDES		Sulfadiazin		50	NS
		Sulfadimidin	100		NS
		Sulfadoxin			NS
OTHERS	Bacitracin		100	50	NS
	Lincomycin		150	100	NS
	Novobiocin		50	25	NS
		Tylosin	50	10-20	NS
		Trimethoprim	50	50	LS

* Results read at first Control Time - after 2.5h of incubation (1, 12)

** Concentrations given in the instructions 2010 (10) – corrected when proved different in our validation (in bald)

NS - Not sensitive

LS - Very low sensitivity

concentration at or lower than MRL (except from Delvotest®SP-NT that detects oxytetracycline at levels two to three times higher than EU MRL).

Specificity

The Twinsensor^{BT} test can distinguish between the tetracyclines and ß-lactams which are the two most commonly used antibiotics in treating lactating cows. It is unable to detect any other inhibitory substance in milk. Delvotest®SP-NT on other hand is sensitive to almost all antibiotic substances (at or below the MRL) used in Slovenia — except of oxytetracycline, dihydrostreptomycin, kanamycin and quinolones, but also detects other inhibitory substances which can be present in milk (naturally occurring inhibitory substances, bronopol, ...) and can give false positive results.

The false positive rate of Twinsensor ^{BT} test was determined as 0%. 69 different blank milk samples

were tested (all detected NEG) and all milk samples spiked with tetracyclines or ß-lactams were all detected correctly as Tetra POS or Beta POS.

Two different blank milk samples were spiked with different concentrations of antimicrobial compounds to which Twinsensor^{BT} test should not be sensitive to and tested. Results are presented in Table 4.

Table 4: Test of the sensitivity of the Twinsensor^{BT} test to other antibiotics than β-lactams and tetracyclines

Compound	500 mgL^{-1}		250 mgL^{-1}	
	BETA	TETRA	BETA	TETRA
Streptomycin	POS POS	NEG NEG	POS POS	NEG NEG
Gentamicin	NEG NEG	NEG NEG	NEG NEG	NEG NEG
Erythromycin	POS POS	NEG NEG	NEG NEG	NEG NEG
Tylosin	NEG NEG	NEG NEG	NEG NEG	NEG NEG
Lincomycin	NEG NEG	NEG NEG		
Enrofloxacin	NEG NEG	NEG NEG		
Trimethoprim	POS LPOS	NEG NEG	NEG NEG	NEG NEG
Chloramphenicol	NEG NEG	NEG NEG		
Sulfathiazol	NEG NEG	NEG NEG		
Bacitracin	NEG NEG	NEG NEG	NEG NEG	NEG NEG
Novobiocin	NEG NEG	NEG NEG	NEG NEG	NEG NEG

POS – Positive results
NEG – Negative results
LPOS – Low positive results
BETA – Beta lactams
TETRA – Tetracyclines

The test reacted Beta POS to high concentrations (500 mgL^{-1}) of streptomycin, erythromycin and trimethoprim and to streptomycin at concentration 250 mgL^{-1}. The Twinsensor^{BT} test is a specific test that detects tetracyclines and ß-lactams in milk samples and can distinguish between those two groups, but can react falsely positive to the high concentrations of some other antibiotics.

Delvotest®SP-NT is a very broad spectrum screening test which can detect almost every inhibitory substance at quite low concentrations but can not distinguish between groups of antibiotics nor between antibiotics and other inhibitory substances.

Robustness

Incubation period

6 different milk samples spiked with penicillin-G (4 µgL^{-1}) and 6 blank milk samples were applied and analysed. The same samples were tested again with the prolongation of the first incubation period from 3 to 6 minutes. Second incubation was not changed. Than the experiment was repeated with the same

samples and only the second incubation was prolonged from 3 to 6 minutes. No change in negative and positive results was observed.

The manufacturer warrants that prolonged first incubation could lead to false positive results. Prolongation of the second incubation period is sometimes needed if the milk sample is very thick and therefore the flow up the test dipstick is slow. The liquid should reach the upper filter paper on the test dipstick to achieve valid results. If you shorten the second incubation, you can obtain false positive results (Tetra LPOS).

When working with Delvotest®SP-NT prolonged incubation can lead to false negative results.

Incubation temperature

The optimum temperature of incubation recommended by the manufacturer of the Twinsensor^{BT} test 40±3°C was changed to 50°C (the only alternative option on HaetSensor). Six different blank milk samples and the same milk samples with standard addition of penicillin-G (4 μgL^{-1}) were applied. Higher temperature of incubation results in false positive reading – all samples applied were Beta: POS.

Incubation temperature recommended by the manufacturer of Delvotest®SP-NT is 64±0.5°C. At temperatures below 64°C, the incubation time was found to increase. At temperatures above the 66°C the blank milk response was found to be positive.

Different analyst

Three different analysts were included in the validation study. Every substance validated was tested at least with two different analysts on different days. No deviation in results when changing analyst was observed.

Different batch numbers

During the validation study of the Twinsensor^{BT} test five different batch numbers of the kit were used. More than three test boxes of each batch number were used and every antibiotic substance was tested with at least two different test bach numbers.

Milk with low pH (spoiled milk)

Two blank milk samples were acidified with hydrochloric acid to pH 4.5. The acidified sample and 2 blank milk samples were tested. Acidified milk gave a Beta and Tetra: POS result at the Twinsensor^{BT} test and a positive response at Delvotest®SP-NT. Two more samples of naturally spoiled milk were applied and gave Beta: POS result at the Twinsensor^{BT} test.

The finding demonstrates that neither of the tests is applicable for the analysis of spoiled milk.

Susceptibility to bronopol (BR)

BR is used as the milk sample preservative (13). The concentration of 0.2% BR in two different blank milk samples was made and applied on both tests. The Twinsensor^{BT} test is obviously not sensitive to BR at this concentration as the results were negative. Delvotest®SP-NT on other hand reacted positive.

Discussion

The Twinsensor^{BT} is a rapid and low cost screening test for the direct detection of tetracycline and ß-lactam antibiotics in milk samples. The test is easy to perform: samples are applied directly; incubation is very short (6 minutes) and the results can be read visually or instrumentally.

In comparison to Delvotest®SP-NT, fewer samples can be done in one step when performing Twinsensor^{BT} (maximum 8 samples), but the incubation is longer at Delvotest®SP-NT (3 hours).

Delvotest®SP-NT detects almost all important antimicrobial substances at or below EU MRLs. Twinsensor^{BT} test on other hand is applicable only for screening tetracycline and ß-lactam antibiotics in milk, but it was seen to have better LODs than Delvotest®SP-NT when detecting tetracyclines in milk samples. For some antibiotics in the group of ß-lactams, the LODs can be low to the point of disturbing, because there are many positive responses even if the antibiotic residues present in the sample are way below the MRLs. Twinsensor^{BT} test is not suitable for detection of nafcillin and cefalexin in milk samples (LOD is higher than MRL).

The most important advantage of the Twinsensor^{BT} test is that the test can distinguish between tetracycline and ß-lactam antibiotics. Delvotest®SP-NT gives us only a positive or negative response.

Neither of the tests is applicable for screening of the spoiled milk (low pH), but both tests were found to be easy to perform and robust in terms of incubation period, different analysts and test batches.

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PREVERJANJE TESTA TWINSENSOR^{BT}, PRESEJALNE METODE ZA UGOTAVLJANJE PRISOTNOSTI ß-LAKTAMOV IN TETRACIKLINOV V MLEKU, IN PRIMERJAVA Z DELVOTESTOM®SP-NT

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Povzetek: Preverjali smo nov kompetitivni receptorski test, imenovan Twinsensor^{BT} in ga primerjali z mikrobiološkim agarsko-difuzijskim testom Delvotest®SP-NT. Oba sta namenjena ugotavljanju prisotnosti zaviralnih substanc v mleku. Pri preverjanju smo upoštevali priporočila odločbe 2002/657/EC, standardov ISO 13969:2003, ISO 18330:2003 in Vodilo za validacijo presejalnih metod (AFSSA Fougères).
Ugotovili smo, da je test Twinsensor^{BT} enostaven za uporabo. Čas inkubacije je zelo kratek (6 minut), metoda je robustna in občutljiva na vse na navodilu proizvajalca navedene β-laktamske in tetraciklinske antibiotike v koncentracijah, ki so nižje ali enake kot so določene s strani Evropske unije (maksimalna koncentracija ostanka antibiotika v hrani - MRL), razen za nafcilin. V primerjavi s testom Twinsensor^{BT} ima Delvotest®SP-NT daljšo dobo inkubacije (3 ure) in je manj občutljiv na oksitetraciklin, po drugi strani pa zazna zelo širok spekter ostalih antibiotikov in zaviralnih substanc, večino v koncentracijah, nižjih od predpisanih MRL vrednosti.

Ključne besede: Twinsensor; Delvotest; mleko; antibiotiki; presejalni test; ostanki; varna hrana

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Review Article

TASTE PERCEPTION: FROM ANATOMICAL TO MOLECULAR LEVEL

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Summary: Taste plays an essential role in food selection and consequently overall nutrition. Our sense of taste helps us to gain information to form a picture of the world by sampling chemicals from our environment. Till now five basic taste modalities have been elucidated: sweet, sour, salty, bitter and umami, however in last years also fatty acid taste is perhaps becoming six scent of taste and existence of more basic tastes is still under debate. Each of these basic tastes has distinct functions. Umami and sweet taste are caloric detectors, eliciting positive hedonic tone, salt taste is important in maintaining sodium levels and is especially important in herbivores, sour taste contributes to recognition of unripe and spoiled food and bitter taste is assumed to detect toxins in the food. We can sense sweet (carbohydrates) and umami (proteins), therefore it would be sensibly to expect that we can sense fat. Each of the taste modalities is eliciting responses through its type of receptors, which are located in different taste buds, on different papillae in diverse areas of the tongue and its surroundings and influencing different nerves to activate taste recognition. Different channels and receptors, including seven transmembrane receptors (7TM receptors) on their own or by group effort, on example heterodimerization, are involved in taste perception by triggering diverse signaling pathways simultaneously in parallel or diametrically. This article reviews all by now known taste modalities from anatomical basis of taste perception till molecular mechanisms.

Key words: taste; sweet; sour; bitter; salty, umami; dietary lipid perception; anatomy; 7TM receptors; channels; taste transduction

Introduction

The sense of taste is a chemical sense for food quality and plays critical role in life and nutritional status of humans and animals. Although sight and chemical sense of smell is very important for food recognition and selection, the final choice of food is made by chemoreception of inorganic ions, sugars, amino acids, peptides and as well xenobiotics and toxins in the mouth, which are all subjected to nutritional chemoreception, followed by adaptive behavior. Taste is important for detecting chemicals in the environment, which directly influence organism, its specific taste receptor cells (TRC) (1).

We enjoy sweet taste, because we have a need of carbohydrates, we crave for salt, because when sodium chloride level is too low in our diet or we call for certain amino acids, which taste sense we entitle umami. On the other hand bitter and sour taste diverts us from most toxins, since majority of noxious substances are bitter and decaying food becomes sour (2). All life forms from bacteria to mammals check its intake by chemoreceptive examination. Already worms, nematode *Caenorhabditis elegans*, distinct between olfaction and taste (3). Both chemoreceptive senses are more clearly separated in arthropods and are distinct in vertebrates. In the fruit fly *Drosophila melanogaster* taste sensations are mediated by nerve cells. Their sensory dendrites are located in “hairs” found on the body surface. Other taste neurons, found on labellum and clustered around pharynx, express GR3 family receptors, belonging to superfamily of seven transmembrane (7TM receptors), as well designated as G-protein coupled receptors (GPCRs) (4). However, the taste receptor cells (TRCs) of vertebrates are not neurons, they have an epithelial origin (5) and are bounded on oral epithe-

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lium, typically tongue, palate and pharynx. On the tongue, the taste buds are located in special folds and protrusions called papillae, which contain large number of specialized bipolar TRCs. TRC express membrane proteins, identified as receptors for bitter, salty, sweet, sour and umami taste.

Taste is unique and can not be mimicked by mixtures of other taste qualities. Whether taste can be described as primary it depends on multiple criteria. Psychophysical and descriptive data isolate one primary taste from another on the basis of statistics, electrophysiological evidence that reports unique neural transduction features on putative taste modality and biochemical and molecular evidence that identifies and localizes unique receptors and cellular responses to the candidate primary modality. And by these criteria five basic modalities have been elucidated: salt, sour, bitter, sweet and umami (6). Through the history of taste research, there were many methods used to understand our taste perception. One of the first was sensory physiology approach that employed methods of psychophysics, initially developed for studying vision and audition and was mainly focusing on discriminating one taste stimulus from another and differences between intensities in distinct subjects. Human psychophysics used three methods to assess taste: absolute threshold measures, recognition threshold measures and suprathreshold measures. In absolute threshold method the lowest concentration of tastant can be detected by a subject as some kind of taste, while receiving three samples, one containing tastant and the others water. Similar method is a recognition threshold method, in which the lowest concentration that subject reports as having specific taste is determined. The suprathreshold measures attempt to quantify taste stimulus intensity, which is complicated by the fact, that perceived intensity of the taste stimulus can vary substantially between individuals (7). In last years animal models have become significant for taste studies. The most commonly used test performed on mainly rats is: two-bottle taste preference experiment, brief access taste assay and operant taste discrimination. In two-bottle taste preference experiment animals receive free access to two bottles, containing water or tastant solution. To measure what is the preference of the animal, the missing volume is measured; however it is important to be aware of strain differences. In the second model - brief access taste assay animals are mildly water deprived and therefore motivated to try one of the multiple spouts presented, which in random order contain a small amount of either water of tastant solution and animal has only a short period of time possibility to sample solution. Every lick of animal tongue is counted for different taste solutions (8). Operant taste discrimination model represents a more direct assessment of the taste by training a standard tastant solution as a discriminative stimulus for food or water reinforcement of an operant task, such as licking water spout (9).

To advance the knowledge of the process of sensation of the taste, knowledge of the sensory organ, the tongue, became more and more important. Therefore studies focused on the anatomy of the tongue, the organization of sensory apparatus and defining physiological-anatomical unit of sensory reception, the taste bud. Furthermore, different forms of receptors have been identified in the taste buds, interacting with the chemical signal - tastant. Next important step was discovery of G-proteins, specifically expressed in the TRCs (10) and later determined as important for perception of sweet, bitter and umami taste. These discoveries lead to assumption that there must be 7TM receptors involved in the process of recognition. 7TM receptors were identified for sweet, bitter and umami taste, although it was found out that for some taste modalities G-proteins are sufficient in eliciting response. First receptor candidates cloned from TRCs, T1R and T2R, were members of class C 7TM receptors. For salty and sour taste diverse mechanisms, eliciting responses through channels were identified (reviewed in 11). Research is now oriented in identifying different signaling pathways through 7TM receptors and new ligands, to either activate or block taste receptors for different signaling pathways. As well in these cases animal studies are present, mostly on genetically modified mice. In studying taste, also genetic approaches and inherited variation in taste abilities studies yielded new information about sense of taste by molecular studies of genes encoding taste receptors and other taste-signaling components. These studies were especially interesting from the fact that for some substances individuals show great differences in their taste thresholds (12). When stimulus activates TRC, receptors are activated, signal transduction cascades are initiated and through synapse and neurons an electrical impulse to the gustatory region of the cerebral cortex of the brain is transmitted, that interprets the sensation of taste.

Anatomy, histology, physiology and map of taste

Tongue lies in oral cavity and is consisted of skeletal musculature, connective tissue, fat tissue, glands and is covered with cutaneus mucous membrane. Mucous membrane helps to block microbes and pathogens from entering the digestive system and helps to moisten the mouth and food. The tongue is able to move in nearly every direction, expand, compress and display a fine degree of articulation. It is important as a tool for consuming and sorting different types of solid and liquid food, influences action of chewing and swallowing, grabbing, palpating, speaking, in animals is involved in fur and skin cleaning and very importantly for gustatory (taste) perception as a carrier of taste organelles (13).

The organ's ability to transform into a variety of shapes comes from its composition of skeletal muscle interspersed with fat. The tongue and its muscles are laterally symmetrical: a median septum divides the organ into two halves. The tongue is consisted of two types of muscles: extrinsic and intrinsic. Extrinsic muscles originate from elsewhere in the body and attach to the tongue. They connect with surrounding bones and help the organ to move up and down, from side to side and in and out. The tongue's extrinsic muscles all end in "glossus," which, unsurprisingly, means "tongue." The genioglossus depresses the tongue and thrusts it out. The styloglossus raises and withdraws the tongue. The palatoglossus raises its back. And, the hyoglossus lowers the tongue's sides. Despite the tongue's fine degree of articulation, the extrinsic muscles also keep it firmly lashed in place. The muscles connect to the mandible, or jawbone, the hyoid bone, a U-shaped structure that supports the tongue, and the styloid processes of the temporal lobes. The styloid processes suspend the hyoid bone with muscles and ligaments, therefore we sort it into the group of bones, that does not come into contact with another. Unlike extrinsic muscles, intrinsic muscles originate within the tongue. They allow it to expand and contract, altering its shape and size. The tongue's intrinsic muscles, which include the longitudinalis superior, longitudinalis inferior, transversus linguae and verticalis linguae, are especially important for speech and swallowing food. The primary blood supply to the tongue is through the paired lingual arteries with return via lingual vein (14, 15). Tongue surface is covered, especially at the palatal and side surface with tongue or gustatory papillae containing taste buds, special ovoid-shaped structures. They are joints of 30-100 small bipolar neuroepithelial cells together with basal and supporting cells and measure (50-60 x 30-70 μm). Total number of taste buds on the tongue is around 4600. Bipolar neuroepithelial cells are also named TRCs. We can find them at low densities on the soft palate, larynx, pharynx, and upper part of the esophagus (Wiggs, 1997).

We differentiate mechanical and taste papillae. Mechanical papillae are divided in filliform, conical and in dogs marginal (they aid nursing to avoid milk spilling) papillae. In the lingual epithelium, taste buds are located in three types of gustatory papillae with different spatial distributions. Taste papillae have different forms and positions and appear in different number and are differentiated in fungiform, circumvallate and foliate papillae. Fungiform papillae cover the front two-thirds of the tongue, are mushroom shaped and have small numbers (1-3) of taste buds on their apical surfaces. On the average, there are 41 taste buds per cm^2 of the tongue, are important for sweet and umami taste sensation and are innervated by facial nerve. The circumvallate papillae are located on the posterior third of the tongue, in the central and lateral regions. They are bigger than fungiform papillae; they do not protrude from the tongue, are separated from surroundings by canal and contain several hundred taste buds. Humans have around 10 circumvallate papillae, whereas rodents have only one, positioned centrally. Each of the papillas is consisted from approximately 250 taste buds, which is 2200 on the whole tongue. Near the circumvallate papillae serose Ebner's glands were found. Ebner's glands are also called gustatory glands and their serous secrete is secreted in the canal surrounding papillas and washing away already tested substances and therefore preparing taste buds for new tasting experiences. They are consisted of TRCs important for recognition of sour and bitter taste and are innervated by glossopharyngeal and facial nerve. Foliate papillae are located at the posterior lateral edge of the tongue and contain several hundred of taste buds. They are mostly reacting on sour taste and are innervated by glossopharyngeal and facial nerve. In humans there are on average 5.4 found in one side of the tongue. Each papilla is consisted froma round 120 taste buds, which are all together 1300 on the tongue. 2500 of foliate papillae can be found on soft palate larynx, pharynx, and upper part of the esophagus. Vagal nerve innervates taste buds in the pharyngeal region (16-18).

The life-span of TRC is around 10 days and every 10 days basal cells, which lay in the vicinity of TRCs differentiate into TRCs. Interesting is the fact that the number of TRCs is decreasing by age. The existing explanation is that every nerve ending can not find new proper TRC in development for the same taste modality, specific for the same type of taste and form new synapse.

Bipolar TRC have two specializations, which are highly important from functional point of view: microvilli, which are in the contact with the oral cavity and synapses with sensory nerve fibers. Taste receptors are mounted on the top of microvilli, working as molecular antennas in the chemical environment. They extend from a small opening, or taste pore, and mingle with molecules of food introduced by saliva. The saliva solution contains digestive enzymes that help to break down foods chemically, which are therefore able to reach receptors. Saliva is secreted by three major salivary glands - the parotid, mandibular and sublingual, as well as other small salivary glands contained within the tongue and mouth. Saliva is also important protector before drying and bacterial infection. Basal part of TRC is connected to fibers of different sensory taste nerves. On the base of TRC afferent dendrites branch into taste buds. When taste molecules bind, receptors trigger transduction cascades that activate synapses and therefore cause excitation of nerve fibers. These carry signal to the brain stream, where central taste processing begins, and elicit responses. The first molecular encounter with tastants by membrane receptors, enables molecular specificity of the taste response and triggers downstream transduction events in TRCs (19).

Neurophysiological studies in several species of mammals have shown four major branches of cranial nerves innervating taste buds and tongue muscles. Taste sensory and muscle innervation is brought to the brainstem by hypoglossal nerve, facial nerve, glossopharyngeal nerve and vagus nerve. Hypoglossal nerve is important for motorical movement of the tongue, while others are important for taste sensation, sense of touch, pain and warmth. Hypoglossal nerve provides motor innervation to the muscles of the tongue (except for the palatoglossus, which is innervated by the vagus nerve) and other glossal muscles and is important for swallowing and speech articulation. First two anterior thirds of the tongue (sensitivity to sodium salts and sugar) are innervated by facial nerve which is consisted from gustatory and sensory fibers. One of the branches of facial nerve is chorda tympani (CT) nerve, which enters through petrotympanic fissure into facial canal towards geniculate ganglion, from where axon enters internal acoustic canal into the cranium till gustatory nuclei. Gustatory nucleus is a part of solitary nucleus (from Latin: nucleus tractus solitarii (NTS)) in the brainstem, laterally from trapezoid bodies. Taste signals to the thalamus, triggers feeding behaviors and via parasimpatic pathway inducing digestive secretions from different glands. It is also important to provide secretomotor innervation to the salivary glands (except parotid) and the lacrimal gland. Posterior third of the tongue is innervated by glossopharyngeal nerve (responding to quinine, acids, weekly to sugars and salts), which is again leading gustatory and sensory fibers till medulla oblongata. Important is lingual branch of glossopharyngeal nerve and is involved in unspecific innervation. Through jugular foramen enters cranium and leads to distal sensory ganglion, consisted of perikarions and furthermore till medulla oblongata. Vagus nerve covers small area on the epiglottis (17, 20, 21).

Receptors and signal transduction in different type of taste

Specialized receptors are stimulated by the chemical makeup of solutions. They respond to several primary tastes: sweet, salty, bitter, sour and umami (savory). 7TM receptors are class C of receptors and are type 1 taste receptors (T1R1, T1R2 and T1R3), type 2 taste receptors (T2R) and taste metbotropopic glutamate receptors (mGluRs) (22). Besides highly important 7TM receptors for taste recognition, mammalian transient receptor potential (TRP) family of ion channels are higly significant for certain types of taste modalities. This is family of ion channels, consisted of 28 members, which are classified into 6 subfamilies: taste vanilloid receptors (TRPV), transient receptor potential cation channel (TRPC), its subfamily M (TRPM), subfamily P (TRPP), subfamily ML (TRPML) and subfamily A (TRPA). Additionally, there are five additional members, referred as PKD1-like family members, distantly related to TRP channels in amino acid sequence. Many TRP channels play important roles in signal transduction in various sensory systems including vision, smell, pheromone, hearing, touch, osmolarity, thermosensation and sweet, bitter and umami taste of diverse animal species, ranging from mammals and fish to fruit flies and nematodes (23). In certain taste modalities firing of action potentials through voltage-

gated Na^+, K^+ and Ca^{2+} channels is highly important, like epithelial Na^+ channels (ENaC), and Na^+ channels susceptible to tetrodotoxin (24). According to the recent literature fatty acid taste, might be a sixth taste, connected to putative CD36 receptor and fatty acid transporter (FAT) (25).

Salt taste

The most abundant dietary source of salty taste is NaCl, which has essential physiological roles in determining blood volume and indirectly influencing blood pressure and water homeostasis. Although salt taste is elicited by many ionic species, Na^+ has a major impact on physiological processes since it represents 90% of all anorganic ions and is therefore the most studied in mammals (12). Salty taste response is also elicited by NH_{4+} and Li^+ and salty-testing KCl that contributes K^+ to the diet. Some of above mentioned ions participate in important physiological processes, such as nerve and muscle signaling, active transport across the membrane and maintaining cell volume, pH and cellular concentrations of other important ions, such as Ca^{2+}(26).

Basis of salt taste perception has been studied for years; however its molecular mechanism is still not fully elucidated. Taste receptors for salty stimuli include several candidates, consisted of specific and unspecific receptors, such as epithelial Na^+ channels (ENaC) and taste variant of the vanilloid receptor-1 nonselective cation channel (TRPV1t) (Lyall, 2004). ENaC is hetero-oligomeric complex, comprised of three homologous subunits (α-, β- and γ), which together act as specific salt-taste receptor by providing a specific pathway for sodium current into TRC, when Na^+ ions are present in the environment in sufficient concentration. Na^+ ions passively flow through these ion channels in the apical, as well as basolateral membrane of TRC according to the concentration gradient and trigger action potential. ENaC channels form adherent junctions on the apical surface of the membrane. With membrane depolarization Ca^{2+} ions enter through voltage dependent Ca^{2+} channels, sensitive to calcium, which elicits neurotransmitter release and signal transmission on primary afferent fiber and eliciting salt taste response (28). ENaCs are distributed in dorsal lingual epithelium in vallate and fungiform papillae. At least one of the subunits of ENaC is under control of hormone aldosterone (29). In animals in sodium-need the sensitivity in sodium taste is increased by induction of more ENaC channels and adapts the tuning of taste acuity in the state of nutritional deficiency. The Na^+ specific salt taste receptor is especially evident in herbivores, where it plays essential role in their foraging for Na^+ (30). EnaCs are sensitive to channel-blocker amiloride and its potent analog benzamil, both diuretic drugs that inhibit Na^+ transport in various epithelial tissues. Amiloride is a guanidinium group containing pyrazine derivative and is known as potassium-sparing diuretic, first approved for use in 1967 (then known as MK 870), used in the management of hypertension and congestive heart failure (31, 32).

Role of ENaC in Na^+ ion transport and specific taste reception was shown by studies on isolated rat and hamster taste buds by showing that amiloride blocks Na^+ current across TRC membranes and that taste nerve responses to NaCl are significantly inhibited by amiloride or its analog. Results revealed that taste responses to NaCl recorded in the afferent CT nerve or in the NTS of various species are significantly inhibited by amiloride without effect on responses to stimuli of other taste modalities (reviewed in 26). Rodents are the species, which are the most sensitive to amiloride and ENaC play important role in perception of NaCl. Since amiloride sensitivity of salt taste is less pronounced in humans, the involvement of other channels was proposed besides ENaCs that may affect NaCl perception (33). It is interesting that TRCs from rat circumvallate papillae in the posterior part of the tongue, innervated by glossopharyngeal nerve give only amiloride-insenitive neural responses to NaCl. However, ENaC can be detected in circumvallatae TRCs.

It was also observed that one of the amiloride or benzamil insensitive salt taste receptors in fungiform papillae taste buds are taste variant of vanilloid receptor 1 (VR1), also designated as TRPV1 (TRPV1t) and are hypothesized to respond to various cations, including Na^+, K^+, NH_4^+ and Ca^{2+}, and therefore described as cation unspecific channels (26). It is one of the non-selective cation channels in nociceptive neurons that mediate terminal pain including the noxious thermal pain produced by vanilloids such as capsacein and resinferatoxin. The amiloride-insensitive component of NaCl CT nerve response, as well as responses to KCl, NH_4Cl and $CaCl_2$ in rat are enhanced by resinferatoxin and capsaicin with increasing concentration up to a maximum enhancement and at higher vanilloid concentrations in neural responses are suppressed (34). The tonic part of the amiloride-insensitive NaCl CT nerve response

are completely inhibited by a TRPV1t inhibitor. The structure of TRPV1t is still undetermined, but it was observed it is constitutively active in comparison to TRPV1 channel, which is not conducting, unless activated by heat, acidic pH or the presence of vanilloids. Also decrease of pH has no effect on TRPV1t, whereas lower pH activates TRPV1. The taste variant TRPV1t cannot detect an increase in food acidity and can therefore function as salt taste receptor, but not as sour taste receptor (35). However, the importance of this protein has been questioned because knock out mice lacking the receptor are nonetheless responsive to salt taste (36). The second proposed option are Na^+ channels, which are susceptible on tetrodotoxin (TTX), which is a neurotoxin found in fish species Tetraodontiformes (pufferfish, porcupinefish, ocean sunfish and triggerfish) (37).

Taste sensitivity to salty stimuli appears to develop postnatally in humans and laboratory rats (38). The hedonic value of NaCl, physiologically the most important dietary salt, varies to some extent with the subject's sodium needs. Salt taste sensation is affected by systemic conditions, that result in increased level of aldosterone and suggests that salt taste reception may involve one of the sodium transporter targets (26). Because salt taste is appetitive, humans ingest more salt than they need. The global high prevalence of hypertension and cardiovascular disease has raised concerns regarding the sodium content of the foods which we consume. Over 75% of sodium intake in industrialized diets is likely to come from processed and restaurant foods. Therefore international authorities, such as the World Health Organization, are encouraging the food industry to reduce sodium levels in their products (39). On the other hand in the state of hiponatremia, Na^+ becomes inaccessible for action potential transmission, which causes hypovolemia and shock and in a rarer cases pathological neurological signs, excitations, convulsions and coma. For this reason it is important to maintain proper sodium concentration.

Sour taste

Sourness is evoked by acids. Sour taste is acceptable or interesting when mild; thereby aiding the recognition of complex food, but it becomes increasingly unpleasant when strong. It serves to detect unripe fruit and rotten food and helps us to prevent tissue damage with acids and problems with acid-base regulation (12). Sources of sour tastants include anorganic molecules such as hydrochloric acid and organic compounds such as acetic, citric, lactic or tartaric acid, which are either natural products of fermentation or basic metabolic pathways such as citric acid cycle. They can be found in most fruits and vegetables, as well as animal products and man-made products, such as wine (40). Limiting the indigestion of acids from food is body strategy to maintain acid-base homeostasis. If sourness is masked by sweet- or salty-tasting substances on example by addition of artificial and natural sweeteners to soft drinks or other acidic beverages, indigestion of acids is tolerable and can be consumed in large quantities. However, by masking sour taste, we ingest large quantities of acids daily, probably more than we are supposed to, given that sour taste is repulsive *per se*. Besides all negative effects for lungs and kidneys, combination of increased acid and sugar in food leads to too low pH in the oral cavity, which promotes tooth enamel demineralization directly and indirectly by encouraging the growth of acid-tolerant bacteria, that are by themselves strong acid secretors (26).

The large variety of mechanisms involved in eliciting sour taste highlights the complexity of taste transduction. A number of candidate receptors for sour stimuli have been proposed, including ENaC, acid-sensing ion channel-2 (ASIC-2), hyperpolarization activated, cyclic nucleotide-gated channels (HCN1 and HCN4) (41). Furthermore, possible candidates would be two pore domain potassium (K^+) channels, which include apical K^+ channel in Mudpuppy necturus (MDEG1), H^+-gated Ca^{2+} channel, proton conduction through apical amiloride-blockable Na^+ channels, a Cl-conductance blocked by 5-nitro 2-(3-phenylpropylamine) benzoic acid (NPPB) and the activation of proton gated channel, BNC-1, a member of the Na^+ channel/degenerin superfamily (reviewed in 12, 24, 42). Sour taste perception is triggered when acidic substances stimulate TRCs, causing depolarization-induced Ca^{2+} entry into TRC (43). Blockade of the H^+-gated Ca^{2+} channels starts depolarization, enables Ca^{2+} ions entrance, which leads to neurotransmitter release and transfer of signal into the primary afferent nerve. To some extent the intracellular pH of TRCs follows extracellular changes in pH, which occurs probably because of the tight junction, which closes the extracellular space of taste bud towards oral space, however is permeable to H^+ ions. The second mechanism is enabled through channels inhibited by amilorid. H^+ ions can use the same channels important for salt and sour taste (12).

In last years two transient receptor potential (TRP) ion channels have gathered strong evidence as putative sour taste receptors and are a focus of additional interest (24). Two receptors - polycystic kidney disease 1-like 3 (PKD1L3) and polycystic kidney disease 2-like 1 (PKD2L1) belong to polycystic kidney disease-like (PKDL) subfamily of TRPs, consisted of 5 members, some of which act as non-selective cation channels and are permeable to both Na^+ and Ca^{2+}. Polycystins (PKD) consist of polycystin-1 (PKD1) and polycystin-2 (PKD2), whose mutations cause an autosomal dominant polycystic kidney disease (ADPKD) (44), one of the most common inherited diseases. ADPKD in humans is manifested with progressive development of fluid-filled cysts from the tubules and collecting ducts of affected kidneys. Association of PKD1 and PKD2 as heteromer appears to be required for formation of a functional receptor that sense mechanical flow, osmolarity and/or unknown extracellular ligands. For both it was shown to be abundantly expressed only in taste tissue and testises (45). PKD2L1 is expressed in all taste areas, while PKD1L3 is expressed only in circumvallate and foliate papillae, but not in fungiform papillae. Both receptors are co-expressed in circumvallatae and foliate papillae, in the same subset of TRCs, distant from sweet, umami or bitter sensing cells, which suggests their involvement in salt or sour taste modality. When studying activation of PKD1L3- and PKD2L1-mediated currents, it was shown that they are delayed in comparison with the onset of sour stimulation (45) and concluded that PKD1L3/PKD2L1 channel has unique off-response property, meaning that the channel is gated open only after the removal of acid stimulus, although initial acid exposure is essential. This type of channel is activated during stimulus application, but not gated open until removal of the stimulus. This could be physiologically significant to enable sour taste sensation regulated by on- and off-response mechanisms. Off-response would be maintained by these described receptors and was also proven in CT nerves. And for on-response other receptors/channels may play their role (24).

Little is known about inter-individual and inter-population variation in sour taste perception and how such variation may be linked to genetic variation. European population was described as fairly narrow in tasting different types of acids (34). Existing twin studies have shown strong heritability component of sour taste sensitivity (46). In future PKD2L1/PKD1L3 could provide a startup for genetic studies for exploring inter-individual variation. Both receptors contain single nucleotide polymorphisms (SNPs) and it is possible that these polymorphisms may affect sour taste perception, but the potential relationship between polymorphisms in these genes, sour taste perception, and subsequent food choices remains to be explored (34).

Neuronal response to all taste modalities consists of rapid phasic burst of action potentials peaking in frequency and is followed by tonic response, which is a rapid decline to pseudo-steady state. Phasic and tonic components of sour taste neural response are well described. The proximate stimulus for sour taste is a decrease in the intracellular pH of a subset of acid-sensing TRCs for week and strong acids alike, which serves as the input to separate transduction pathways for the phasic and tonic parts of the sour neural response. This causes a shift in the cytoskeletal F-actin to G-actin, equilibrium in the G-actin direction, resulting in cell shrinkage, which was also observed from imaging studies of fungiform papillae. This activates acid-sensitive shrinkage-activated nonselective cation channel (SANSCC) in the basolateral membrane of TRCs that results in cell depolarization and leading to phasic neural response. SANSCC is involved in eliciting the phasic part of the CT nerve to acidic stimulation. In the subset of TRCs a decrease in pH induces an increase in intracellular Ca^{2+} concentration, which is necessary to sustain tonic phase response. Ca^{2+} ions activate basolateral Na^+-H^+ exchanger isoform 1 (NHE-1), which is responsible for pH and cell volume recovery and for the observed level of neural adaptation (tonic response) in CT nerve in response to acid stimuli (26). In support of this mechanism, complete elimination of the phasic response is achieved by disrupting the depolarization of F-actin to G-actin, which was performed in rat tongue with cytochalasin B and furthermore restored by treating rat tongue with phalloidin, which binds to F-actin and stabilizes the actin cytoskeleton (47). To prove that Ca^{2+}-activated NHE1 represents the molecular basis of TCR sour adaptation; it was published that by increasing taste cell intracellular Ca^{2+} *in vivo* by lingual application of ionomycin increases the level of neural adaptation and decreased tonic response level to an acidic stimulus (48). Adaptation to sour arises from the activation of the basolateral sodium-hydrogen exchanger isoform-1 by an increase in intracellular calcium that sustains the tonic phase of the sour taste response.

Bitter taste

Bitter taste is bearable when week and therefore helps us to recognize complex food, however when strong, becomes repulsive and has strong negative hedonic tone. Bitter taste is effective warning that we should not use potentially dangerous ingredients. Therefore one of the important and interesting challenges in bitter taste research is to understand how the receptors involved in recognition of bitter taste have formed during evolution to serve this mission. Many organic molecules, originating from plants are bitter, including caffeine, nicotine, strychnine, as well as industrial drugs (49). Around 10% of plants may contain toxic glycosides or alkaloids, which are in plants chemical defense systems against herbivores and pathogens. Also insects can synthesize cyanogenic glycosides for their defense (50).

Searching through databases has revealed that 7TM receptors, with short amino-terminal domain, comprised from at least 40 members, designated as T2R family lie on mouse chromosome 6, near the locus for bitter taste (51) and are expressed on the subset of specific TRCs on the front thirds of the tongue and palate epithelium. At least 24 of these receptors are involved in responding to bitter agents. First deorphanied was murine T2R5 (mT2R5) by responding to cyclohexamide in brief access taste aversion assay. It was shown that a single taste TRC expresses a large repertoire of T2Rs, suggesting it has ability to recognize multiple tastants, however single taste nerve fibers carries signals that discriminate between bitter compounds (52). It was also found out that mouse strains vary in sensitivity to specific bitter substances, such as cyclohexamide and sucrose octaacetate, and is connected to genetic variation on chromosome 6. Bitter taste seems to be the most complex taste quality in humans, based on the variety of chemical structures that elicit bitterness and on the large number of genes, encoding receptors for this taste modality. Bitter taste genes were designated as T2R or TAS2R genes. In humans there are 24 potentially functional T2R genes and several T2R pseudogenes, which differentiate between each other in 25-89% amino acid residues, and reside on three different locations (12p13, 7q31 and 5p15) (12).

Interesting for bitter taste modality is that responses of humans to some bitter compounds show a bimodal distribution that distinguishes two phenotypes, tasters and non-tasters and the compounds the most studied in this respect are phenylthiocarbamide (PTC) and similar 6-n-propyl-2-thiouracil (PROP), since some of the population taste it as bitter and some of the population is "taste blind" for PTC. Initial studies have shown that ability to detect PTC was inherited by classic recessive Mendelian mode of inheritance. Further genetic differences for T2R bitter receptors were provided by the variable ability in humans to intensively sense bitter taste of PROP and PTC. On this basis taste subjects have been identified as non-tasters, tasters or super-tasters according to the intensity of their responses to substances (53). The variation in taste sensitivity was mapped to chromosomes 5 and 7 and differs at 3 amino acid positions. Later on human candidate receptor for these substances were cloned, designated as T2R38 and was responding to both, PROP and PTC. Additionally, variation of human threshold sensitivity to test these substances was linked to mutations in the gene for receptor (12). In humans also T2R16 was identified by calcium signaling assay as the receptor that mediates bitter taste (54) and furthermore more receptors in different species were cloned. Later also 3 other forms of this gene were observed, mostly in sub-Saharan African populations (12). For these genes it was demonstrated that they show a broad range of variation, including a substantial number of SNPs and many of them are non-synonymous and change amino acid encoded in the protein, which is important for elucidating important sites for bitter transduction within these proteins (12, 55). The non-tester alleles reside on a small chromosomal region identical by descent, indicating that non-tasters are descended from an ancient founder individual and consistent with an origin of the non-taster allele preceding the emergence of modern humans out of Africa. The two major forms differ from each other at three amino acid positions and both alleles have been maintained at high frequency by balancing natural selection, suggesting that the non-taster allele serves some function on example serve as a receptor for another, yet unidentified toxic bitter substance (55). 75% of individuals worldwide perceive PTC intensely bitter, while to others it is relatively tasteless and this difference is stable over lifetime of a given individual (12).

Data obtained from *in situ* hybridization showed that one TRC expresses a huge repertoire of T2Rs, which shows that every TRC is capable of recognizing more taste modalities. Members of T2R family have been found co-expressed with $G\alpha_{gustucin}$. Mice

models with knock out gene for $G\alpha_{gustucin}$ have shown lower sensitivity for bitter substances and as well for sweet substances, such as saccharin and sucrose. Bitter compound binds to T2R $G\alpha_{gustucin}$ and amplifies the signal, which leads to activation of intracellular phosphodiesterase (PDE), which lowers the activity of cyclic adenosine monophosphate (cAMP), interrupts normal cation release through channels that act through cAMP leading to cell depolarization. These complex events lead to transient elevation of cyclic guanosine monophosphate (cGMP) (56). The second signal transduction cascade is generated through phospholipase C-inositol trisphosphate (PLC-IP_3) activation system. Bitter tastant stimulates 7TM receptor, which activates PLC that leads to IP_3 release, and Ca^{2+} ions release from intracellular storage and subsequently to depolarization and neurotransmitter release on the afferent nerve fiber. Also βγ subunit of the heterotrimer protein gustducin (G $\beta_3\gamma_{13}$) is able to activate PLC $C\beta_2$. It is interesting; however still unclear, that both pathways are activated simultaneously, whether this is needed for bitter taste recognition or it is just parallel amplification (57). It is known that human Gγ13 is participating in bitter taste signaling (55, 58).

Interesting is the finding that some bitter peptides with amphipatic properties do not need 7TM receptors, they interact directly with G-proteins, like quinine (59). General structural characteristics of hydrophobicity and hidrophility enable compounds to rapidly insert into cell membranes where they directly activate G-proteins or other signaling molecules independent of receptor occupancy. Quinine activates $G\alpha_{transducin}$ and $G\alpha_{i/o}$ proteins *in vitro*. The second example is caffeine and other metilksantins, which penetrate cell membrane and block intracellular PDE. In both, further signalization could be under the control of nitric oxide, since nitric oxide synthetase was found in TRCs (60).

Transgenic animals deficient of critical components of bitter receptor signaling pathways still avoid high concentrations of the bitter compounds denatonium benzoate and quinine (61). One of the animal experiment regarding bitter taste has shown that mice engineered to express bitter taste receptor for β-glucopyranosides in »sweet cells« become strongly attracted to bitter compound, showing that the taste of bitter or sweet compound (that is, the perception of sweet and bitter) is reflection of the selective activation of T1R-expressing vs. T2R expressing cells, rather than a property of the receptors or even tastant molecules (49).

Sweet taste

Sweet taste is strongly pleasant and it corresponds to soluble carbohydrates, which are present in sufficient concentrations in the oral cavity. However, a wide diversity of non-carbohydrate molecules is also sweet. Extensive research has been made to define characteristics of "sweet" molecule and its "sweet" receptor, to be able on the basis of existing binding models, predict new high-potency sweeteners. First 7TM receptor identified, involved as being a candidate for trehalose receptor was found in *Drosophila* (62). Further on it was found out that in mouse genome receptors for sweet taste are located on the chromosome 4, in two taste-related locations, the *Dpa* and *Sac* locus (63). Mutations in the Dpa locus resulted in a partial loss of taste acuity for the sweet amino acid D-phenylalanine, whereas mutations in the Sac locus caused partial loss of taste acuity for sucrose, saccharin and other sweeteners. The chemical structure of substances that taste sweet is almost as broad as the set of compounds that taste bitter, from natural (sugar, glycerol, amino acids, aspartame, thaumatin, monellin) to whole set of artificial sweeteners (Na saccharin, Na cyclamate, dulcin and Pb and Be salts).

Inter-individual differences in response to sweet compounds are not yet fully characterized and intersubject differences are relatively modest. The names for associated genes for sweet receptors are *Tas1r2* and *Tas1r3* in mice and in humans *TAS1R1* and *TAS1R2* and were delineated through gene-mapping experiments in mice and humans (12). Furthermore, it was expected that more genes for perception of sweet taste would be found near these locuses and strategy was shown to be successful. 7TM receptor with large terminal domain (T1R3) was found (64), similar to T1R1 and T2R, described previously (65).

T1R1 was found on the buds of anterior, lateral and posterior tongue and in the same TRCs that express T1R2, suggesting they might elicit response by forming heterodimers (66). Since *Tas1r3* gene is the only 7TM receptor coding gene at Sac locus, therefore its product T1R3 is a strong candidate for a sweet receptor, practically confirmed by observations in mice, that differ in taste-ability also differ in several point mutations in *Tas1r3*, displayed mainly as decline in function. Finally, when T1R3 was expressed in oocytes, it was shown that receptor does not respond to sweetners by its own, just after coexpression with T1R2, showing that receptors functionally work as heterodimers and as well shown as

first functional sweet receptor found in mammals as a heterodimer (67).

Interesting is the observation that receptor for sweet taste, functionally being heterodimer, is also often written as T1R2/T1R3. As Class C 7TM receptor member is unique in the case of N-terminal Venus flytrap-like domains (VFDs). Like in the metabotropic glutamate receptor also T1R2/T1R3 receptors are likely to bind sweeteners in the VFD on T1R2 (aspartame and artificial sweetner neotame), however cyclamate binds within the 7TM domain of T1R3. T1R2/T1R3 heterodimer is the first functional 7TM receptor unit demonstrated to have more than one agonist binding site (orthosteric sites) (68). This leads to further questions whether VFD on T1R2 is perhaps ortosteric site for sucrose and other carbohydrates. Kniazeff and al. (69) has demonstrated that both VFDs of the homodimeric metabotrophic glutamate receptor must be populated by glutamate to give a maximal response, however in the other representatives of family C 7TM receptors γ-aminobutyric acid type B ($GABA_BR$), GABA binds only to one receptor type. Therefore more options of dimer activation by ligands exists, one is that sucrose and other sweeteners bind to the VFDs of T1R2, they might also bind to VFDs of T1R3, second would be that VFDs are different and they can bind just to one or two carbohydrate sweetener molecules in each VFD and leading to high state of activation. Also it is not known whether there is synergy existing between different sweeteners on example aspartame and cyclamate and since they bind on separate orthosteric sites they could have cooperative binding effect. Given that there is also synergy existing between saccharin and cyclamate, it is possible that more orthosteric sites exist (68).

Sweet taste receptor needs many G-proteins. Especially important is $G\alpha_{gustucin}$, which is besides for sweet perception important also to percept bitter taste. $G\alpha_{gustucin}$ is active through adenyl cyclase (AC) and cAMP through K^+ ion channels at the basolateral side of membrane. T1R3 is expressed in 20% of the TRCs, some of which also express $G\alpha_{gustucin}$. Data from knock-out mice showed that co-expression of both is compatible, with a role in $G\alpha_{gustucin}$ in sweet taste (70). Signal transduction in sweet-responsive cells is complex and questionable. At least two pathways have been described, one mediated through cGMP or cAMP and the second through elevating the level of IP_3, as second messengers.

On the apical membrane of TRCs are receptors binding glucose, sucrose or other carbohydrates. Transduction mechanism runs through the blockage of K^+ channels. Binding of sugar on the receptor activates AC, which leads to elevated level of cAMP and furthermore with protein kinase A (PKA) activated phosphorylation of K^+ ion channels and inhibits them. After depolarization Ca^{2+} ions enter the cell with depolarization of activated Ca^{2+} channels, leading to transmitter release and further to transmission of the signal. It was thought that inhibition of K^+ conductance was occurring through PKA, but cyclic nucleotide-gated channel (CNGgust) was found in TRCs, important for membrane depolarization and Ca^{2+} inflow, when cAMP increases.

It was shown that sugars activate cyclic nucleotide cascade, leading to an increase of cAMP, membrane depolarization and Ca^{2+} uptake, whereas non-sugar sweeteners activate IP_3 cascade in the same cell (71). Membrane depolarization by inhibition of K^+ conductance may be a common feature for both pathways. An increase in the cytosolic Ca^{2+} concentration occurs in both pathways; even the source of Ca^{2+} ions is different. It looks like there is variability in utilizing different pathways across the posterior and anterior part of the tongue and across sweeteners in animal species.

The second pathway through IP_3 causes intracellular release of Ca^{2+} ions. Released Ca^{2+} ions enable neurotransmitter release. This group includes artificial sweeteners, such as saccharin, which is sweet only to human, but not to bees and butterflies, cyclamate and aspartame, which is a combination of two natural amino acids – asparate and phenylalanine and it is 2000-fold sweeter than sugar. In this group we can also include sucralosa, which is a chloride, including carbohydrates and it is 600-fold sweeter than sugar. Also lead and berilium salts are sweet. It was shown in hamster that PKA inhibitors do not inhibit sugars-sweet response in the posterior part of the tongue; on the contrary they accelerate it, which shows that PKA is not directly involved in the response to sugars, but may be involved in the adaptation. On the contrary inhibition of PKC did not affect responses to sucrose, but inhibited responses to artificial sweeteners, which showed that transduction of two kinds of sweeteners differs. Inhibition of the cAMP enhanced the responses to sucrose but not to synthetic sweetners, indicating that Ca^{2+} ions release during stimulation with synthetic sweeteners may depress a simultaneous response to sucrose by activation of this enzyme (72, 73).

Sweet taste is modified by circulating hormones. Leptin, a protein hormone (reviewed in 74) has gath-

ered much interest on sweet-responding cells. Leptin is secreted mainly by adipocytes and regulates body mass. A full length leptin receptor is expressed in various tissues and among others also in TRCs and it suppresses insulin secretion by activation of ATP sensitive K^+ channels. Its inhibitory effect on TRCs also involves the activation of a K^+ conductance and membrane hyperpolarization (75). Thereby the hormone partially blunts nerve signals indicating sweet taste, which presumably makes food less attractive. During the starvation the production of leptin is decreased and the resulting disinhibition in the target tissues diminishes energy expenditure and leads to motivational state of hunger. At the same time, disinhibition of sweet-responsive TRCs enhances sensitivity to sweet taste and makes food more attractive and therefore supporting its role in whole organism (63).

Umami taste

Umami – the "meaty" taste of glutamate and some other L-amino acids is dominant flavor of the food, which contains L-glutamate, an amino acid that is abundantly found in food and often occurs as monosodium glutamate (MSG), consisted from two tasting stimuli: Na^{2+} ions and glutamate. L-glutamate guides the intake of peptides and proteins, from which it is released by proteolysis (curing and decay). Animals are attracted to this taste. The characterizing taste is enhanced by purine nucleotides 5'-ribonucleotides such as inosine 5'-monophosphate (IMP) and guanosine-5'-monophosphate (GMP), which are also present in decaying tissues, that is why some people misconcepted that glutamate contained food might be harmful (77, 78). L-glutamate is a cleavage product of all proteins. The synergism between MSG and the nucleotides was explained by an allosteric effect (79).

Type of food with characteristic umami taste is typically chicken broth, meat (beef, pork and chicken), seafood (fish, oyster, crab, sea urchin, various sea-weeds and others) and aging cheese, however it is also find abundantly in a wide array of vegetables, such as tomatoes, potatoes, mushrooms, carrots, cabbage, soybean and green-tea (80). MSG is added to different sorts of food as a taste enhancer and is the main ingredient of soy sauce and Japanese soup base. It is interesting that taste of boiled crab meet can be reproduced by mixing amino acids: glycine, alanine, arginine, MSG, monophosphate disodium salt (IMP) and salts in particular ratio. When umami constituents are eliminated, the characteristic taste of crab disappears, suggesting that umami substances are essential for producing the unique taste of many foods (81). When umami substances are added to food they enhance food palatability (76). Other amino acid that trigger umami taste is L-aspartate (82), showing umami substances are originally acids, therefore at neutral pH they exist in the salt form. Usually they are sodium salts, i.e. glutamate, disodium inosinate and disodium guanylate. Thus the umami substances contain the sodium ion (80).

Umami taste was the first time identified by Prof. Kikune Ikeda in Tokio more than 100 years ago in 1909 (83), however it was translated in English in 2002 by Ikeda (84). Umami, a term describing meaty, savory flavor, derives from the Japanese *umai* (delicious, good taste) and designates pleasant taste sensation, which is qualitatively different from sweet, salty, sour and bitter taste (84). It was hard to accept this new taste modality, since this taste is mild even in high concentrations of tastants and especially because the umami taste from anionic L-glutamate, was difficult to dissociate from the cationic sodium, which forms salty taste and is also found in MSG (12). However, the umami substances L-glutamate, IMP and GMP are still an object of interest and their taste responses are investigated in humans and animals. Therefore unique taste of umami argues for a specific receptor at taste level. The taste synergism between MSG and certain 5'-ribonucleotides provides a pharmacological mechanism showing that several receptors are involved in umami taste recognition.

It was also discussed whether MSG and umami are the same. It was concluded that since umami was described as delicious, nice and palatable and MSG by itself does not in any sense represent deliciousness, on contrary being rather unpleasant, bitter and soapy, MSG and umami can not be unified. However, when MSG is added in low concentrations to different foods, the flavor, pleasantness and acceptability of food increases, which is a perfect example of distinction between the taste of single testant and the effects upon flavor of tastants in food (85). Two hypotheses seek to explain umami taste transduction through 2 categories of receptors: stimulus-gated ion channels (*N*-methyl-D-aspartate (NMDA)-type glutamate ion channel) and 7TM receptors (truncated and brain forms of metabotropic-type glutamate receptor: mGluR4, mGluR1 and brain forms of mGluR4 and mGluR3, as well as

other 7TM receptors: T1R1 and T1R3 (86). Na^+ ions use separate way of eliciting their response. Umami taste is very different perceptually from sweet taste; however they are closely related phylogenetically. The names for associated genes for umami receptors are *Tas1r1* and *Tas1r3* in mice and in humans *TAS1R1* and *TAS1R3* (12).

In rat fungiform papillae through EnaC Na^+ ions cross, however MSG crosses through metabotropic and ionotropic receptors. ENaC are not directly involved in glutamate signal transduction, however co-localization with glutamate receptors enables substrate to integrate through this pathway. Initial results in support of the glutamate-stimulated ion channels have shown they could be reconstituted into lipid bilayer and that the addition of mM concentrations of L-glutamate led to an increase in conductance of bilayer (86). Further studies monitored intracellular Ca^{2+} and membrane voltage in isolated TRCs from mouse vallate and foliate papillae. Cells responded to L-glutamate with either increase or decrease in the intracellular calcium and membrane depolarization accompanied to increase in the intracellular Ca^{2+} (86). These results show more receptors that activate different pathways exist. There are 2 types of glutamate receptors – stimulus gated-ion channels, which are stimulatory and metabotropic channels. Ionotropic glutamate receptors, connected to ion channels, induce signal transduction by altering ion flux through an ion channel directly coupled to and gated by glutamate binding site. These receptors can be delineated by differential sensitivity to glutamate analogs such as a-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA), kainic acid and *N*-methyl-D-aspartate (NMDA) (86, 87). NMDA receptors are integral receptor non-selective cation channel complexes. When stimulus binds to receptor site on the channel complex and therefore directly gates an ion channel, allows influx of cations - Na^+ and Ca^{2+} ions in the TRC, which leads to the depolarization of TRC. This depolarization induces further modulation of voltage-sensitive channels, leading to cellular depolarization, in the basolateral region of the TRC, sustaining and increasing depolarization sufficient to induce neurotransmitter release.

7TM receptors in which glutamate binding induces changes in intracellular messengers and then alter the balance of intracellular ions were shown to play an important role in umami signal transduction. At least two types of 7TM receptors have been recognized till now. Studies have shown that a subset of TRCs contains metabotropic glutamate receptor (mGLuR4), which differs from brain version in truncated N-terminal domain (NTD), suggesting an important adaptation to high glutamate concentrations occurring in the food (88). The metabotropic receptors are classified in several groups, i.e. I. (mGluR1, mGluR5), II. (mGluR2, mGluR3) and III. (mGluR4, mGluR6, mGluR7 and mGluR8) (89). Umami involved should be mGluR4, mGluR1, brain mGluR4 and mGluR3. Chaudari (90) has reported in 1996 that mGluR4 is expressed in rat papillae-bearing taste buds and suggested that mGluR4 might be a chemosensory receptor responsible for umami taste (Chaudahari, 1996). Binding of MSG on these 7TM receptor activates $G\alpha_{i/o}$ protein, which decreases cAMP by inhibiting the action of AC. Lower levels of cAMP result in a lower activity of PKA, decrease in Ca^{2+} ions and inhibition of voltage sensitive ion channels on the basolateral membrane, bringing about no charge or hyperpolarization of the cell (86). CHO cells transfected with taste-mGLuR4 were responsive to L-2-amino-4-phosphonobutyrate (L-AP4), ligand that elicts umami taste responses in humans and MSG in concentrations similar to the ones that elict umami response *in vivo*. However, it was still apparent that taste mGLuR4 are not the only receptors important for umami taste. One of the important evidence was that taste m-GluR4 receptors lacks a portion of the domain, necessary for glutamate recognition and that mGluR4 knock out mice still respond to umami stimuli (9). It is also possible that ion channel receptors or other 7TM receptors would act in concert, with the mGluR4 providing inhibitory signal in some cells to enhance the contrast with excitable cells. The interesting option is to consider analogy with visual system, where activated cell inhibits surrounding cells, to enhance visual acuity. By this possibility mGluR4 inhibitory response may signal on example through Merkel-like cell, which are also present in taste buds of animals, which would as in visual system transform the initially inhibitory signal into an excitatory one (92). The mGluR4 receptor was originally found in the brain, where it responds to extracellular glutamate by downregulating cAMP. This receptor is expressed on presynaptic terminals of both glutaminergic and GABAnergic neurons, where it mediates glutamate-dependent regulation of neurotransmitter release. In addition mGluR4 is expressed in TRCs, making it a candidate for umami receptor. Glutamate activates mGluR4 at μM concentrations far below the threshold; however alternative transcript mGluR4 variant, with truncated N-terminus can transduce

a response to glutamate. It is actually surprising, since it is known that in family C 7TM receptors are forming N-terminal VFDs (69), where glutamate binds. Alternatively the answer could lie in the receptor additional binding site. This triggered issues whether mGluR4 was really the right receptor. However, evidence has shown by confirming activation of receptor by agoinst L-AP4 and *in situ* hybridization of mGluR4 in the TRCs, that 40% of receptor is expressed in TRCs. Another question which appears is how decrease of cAMP can modulate membrane potential and cause TRC to signal. By electrophysiological experiments it was found out that 60% of TRCs respond to glutamate with sustained hyperpolarization and just 4% of cells respond with transient depolarization, which looks like sustained hyperpolarizing response is what leads to taste signaling. By this model glutamate triggers decrease in cAMP, resulting in the closure of cyclic nucleotide-gated channels and hyperpolarization of TRCs. Since it was shown that MSG induces a large response in the taste nerve, it was postulated that a receptor for umami taste should be an excitatory receptor and therefore speculating mGluR4 can not be a receptor for umami taste or at least the main receptor for umami taste, although L-AP4 has an umami taste (80).

More recently other receptors, members of 7TM receptors were discovered and molecular methods have shown that umami processing seems to be closely related to sweet taste processing at the molecular level. 7TM receptors, T1R1 and T1R3, cloned both from humans and rats appear to form a heteromeric umami taste receptor. Co-expression of T1R1 and T1R3 responded exclusively to umami L-amino acids, such as L-glutamate in rodents and specifically to L-glutamate in humans. Human and rodent receptors show strong synergy when co-treated with IMP or GMP (93). In mice this heteromers responds to many amino acids contained in the food, but in humans its response is preferentially to L-glutamate and is enhanced by IMP (94, 95), which perhaps reflects differences between two species in their natural diets. Expressed singly, the T1Rs express weakly, if at all to tastant *in vitro* (94). T1R1/T1R3 heterodimer is coupled to a G-protein, consisted from Gα subunit, that modulates cAMP levels and Gβγ subunit that stimulates PLC. Through Gβγ transduced part of the pathway appears to be necessary for umami transduction and therefore considered as dominant pathway (96). Upon receptor binding Gβγ stimulates $PLC\beta_2$, causing production of second messengers IP_3 and DAG. IP_3 causes release of Ca^{2+} from intracellular stores and Ca^{2+}-dependent activation of monovalent cation channel, TRPM5. This leads to membrane depolarization, action potential generation and release of transmitter, believed to be ATP (97). Evidence for this hypothesis comes from molecular and immunocytochemical studies, showing that relevant effectors are co-expressed with T1R1/T1R3 (93). It is interesting that Gα subunit that couples to T1R1/T1R3 heterodimer differs with respect to taste fields. In fungiform and palatal taste buds receptors are co-expressed with $G\alpha_{gustducin}$ and/or $G\alpha_{transducin}$, however in vallate and foliate taste buds Gα associated with T1R1/T1R3 has not been identified, but decrease in cAMP suggests the involvement of $G\alpha_i$. (93). It was also reported that umami taste responses are mediated through $G\alpha_{transducin}$ and $G\alpha_{gustducin}$ in anteriorly placed taste buds, however TRCs at the back of the tongue respond to umami compounds independently of these two G-protein subunits (98, 99).

Results based on reports from cDNA library derived from rat vallatae papillae, *in situ* hybridization studies have pointed on mGLuR4 family of receptors. Use of agoinst of mGLuR4 receptor L-AP4 in patch recording studies displayed a transient inward current, induced by glutamate (100). Biochemical studies to characterize receptor for glutamate were performed on membrane preparations from bovine circumvallatae papillae, where L-gutamate binding was observed and as well enhanced by 5'-ribonucleotides. First *in vivo* data were performed on Scl:ICR strain of mouse, which was able to discriminate MSG from other basic tastes. Information was based on information from glossopharyngeal nerve, since fibers of the nerve were uniquely sensitive to MSN (100). The physiological roles of these receptor heterodimers was established in studies with transgenic mice. *Tas1R1* and *Tas1R3* knock out mice were generated and the sensitivity to either umami or sweet taste was compared to results obtained in cell-based assays. Mice were characterized through behavioral tests and by measuring activity of the gustatory CT nerve after exposure to different taste stimuli (101). As expected, results have shown that *Tas1R1* and *Tas1R3* knock out mice showed a complete loss of preference for umami, since they have exhibited no CT nerve activity after stimulation with glutamate and clearly showed that umami taste is preceded by heteromeric T1R1/T1R3 receptor (94, 95). Another study on dogs showed that not every dog responded to 5'-oligonuclotides and that sensi-

tivity to MSN varied. In dogs, CT nerve is sensitive to MSG and 5'-oligonuclotides, such as guanosine-5'-monophosphate (GMP), since AMP was effective only in beagles (102). The same nerve is also sensitive in chimpanzees, however further representations for umami stimuli were localized in orbitofrontal cortex. Nevertheless, species, strain and individual differences were observed, however the basic postulates remained the same. Furthermore, both, kinetics of the binding data and *in vivo* data suggested that GMP increased number of binding sites for L-glutamate or increase in affinity for L-glutamate as a result of nucleotide interaction with a closely associated site proposed was observed and therefore an allosteric-type model for MSG/5'ribonucleotode binding interaction was proposed (79).

Human variability of umami taste is still poorly understood. In European adults responses to L-glutamate have been tested and only 27% subjects were unable to distinguish MSG vs. NaCl and were therefore unable to distinguish salty umami taste component from the salty component of MSG, which suggests reduced ability to taste umami (103). Regarding genetic basis underlying these mechanism, sequence of *Tas1R* gene was compared between different populations (e.g. Asian, African, Auropean and others) and several SNPs were identified within extracellular domain of *TAS1R1* and *TAS1R3* and their frequencies varied between populations suggesting interindividual variability (104). Regarding taste enhancement with IMP it was found out by psychophysical method that taste enhancement occurred when IMP was added to several sweet amino acids, such as L-alanine, L-serine and glycine. The enhanced quality of taste was recognized as umami and was not blocked by the sweetness inhibitor. The connection appears to exist through T1R3 subunit, which is shared with sweet taste receptor (75).

Is there a fatty acid taste?

Fatty foods are very palatable and most people prefer high-fat food, such as ice cream, hamburgers, steaks and mayonnaise to low-fat food, although that dietary fat is tasteless. It is interesting that although people can not feel the taste or smell of dietary oil and fats clearly, fat is interestingly tasty. Neuropeptides and neurotransmitters, related to hedonic or aversive response in the brain are released after basic tastants, described under five basic tastes and accepted by taste receptors in the TRCs. Paradoxically, dietary oils and fats do not stimulate the taste in the classic sense of tasting, however recently some resemblance to other taste modalities has been described (19).

Obesity is recognized as a worldwide health problem and overconsumption of fatty foods significantly contributes to this phenomenon. Therefore gaining knowledge about molecular mechanisms of fat preference and overeating might help to lower the risk of obesity. The disturbing data is also that chronic high-fat diets promote greater daily intake by eliciting larger and more frequent meals and increase the risk of obesity. When lipids indigested, they trigger set of regulatory events that limit food intake. Lipid-mediated regulation of food intake results from integration of multiple short-acting early (oral) satiety signals and long-acting, delayed (postabsortive) homeostatic signals. Early events are consisted from olfactory, textural and gustatory cues. Olfactory information is mediated through olfactory nerve, texture of foods through trigeminal nerve and gustatory information via the facial (branch CT nerve), glossopharyngeal and vagus nerve. A key early regulator is cholecystokinin (CKK), which is released by proximal intestine in response to dietary lipid loads and is sending meal-reducing signals through vagal afferent pathway, which express receptors for CCK, designated as CCK1R. A short-term satiety agent include glucagon-like protein 1 (GLP1) and peptide YY (PYY), released by ileal enteroendocrine cells in response to fat (105). Delayed events are associated with postindigestive and postabsortive signals. Postindigetsive/absorptive information via nerves converge on the NTS in the brain steam that connects to central regulatory areas like nucleus accumbens (Nac) and hypothalamus (HT), both of which are constitutes of metabolic and pleasure pathways. The NTS also projects efferent nerves toward indigestive tract, which accounts for the cephalic phase of indigestion, triggered by oral lipid stimulation facilitating fat digestion and absorption. HT activity is modulated by plasma factors (hormones, regulatory peptides, lipids). Satiation, which largely determines the size of meals, mainly depends on postidigestive signals. Postprandial satiety is largely responsible for meal frequency and essentially related to post-absorptive signals. Alipoprotein A-IV (ApoA-IV) promotes satiety and it was shown in rats that peripheral or cerebroventricular injections decrease food intake in dose-dependent manner and it becomes less efficient when subjected to chronic high-fat diets. Long-term satiety agents include leptin, which is produced by adipose tissue (74). It was shown that

high levels of leptin during obesity might contribute to reduce satiety sensitivity observed during chronic exposure to fatty foods. Such reduced satiety might help to explain the overfeeding frequently found in obese animals and humans. Although there are several factors in blood preventing high foods intake, it was shown that free fatty acids (FFA) can modulate feeding behaviors through direct actions on the brain through acting on the ion channels or binding to specific receptors in fatty acid (FA)-sensitive hypothalamic neurons (106). »Fat taste« perception was supposed to be evolved from evolutionary perspective to detect high energy foods and to select foods containing fat soluble vitamins and essential FAs (107). Important function of fat detection in cephalic phase would be to aid digestive system for lipid metabolism. It was seen that both rats and mice select high fat diet over a low-fat diet. Since preference on low or high-fat diet is based on animal instinct, using laboratory animals to get new insight is relevant (19). Rodents, like humans display preference for lipid-rich foods and therefore provide useful models to explore the mechanisms of fat preference and also overeating. The mechanisms guiding fat detection have traditionally been attributed to texture and olfaction, however also oral detection is very important. First "fat taste" receptor evidence have come from evidence that FAs, specifically unsaturated long-chain fatty acids (LCFAs) were prolonging cell depolarization by influencing K^+ channels on TRCs (108). Candidate for "fat taste" receptor is proposed to be an oral lipid sensor CD36 (107).

CD36 is a receptor-like protein that binds saturated and unsaturated LCFA with affinities in nM range and has structural and functional features required for putative taste-based lipid receptor. It belongs to family of class-B scavenger receptors. It increases uptake of LCFA by cardiomyocytes and adipocytes and uptake of oxidized-low-density lipoproteins (LDL) by macrofages, it modifies platelet aggregation by binding to thrombospondin and collagen, facilitates phagocytosis of apoptotic cells by macrophages and plays role of taste reception of dietary lipids on the tongue (106). CD36 is an integral membrane protein creating large extracellular hydrophobic loop (likely the site interacting with FAs) and two short cytoplasmic tails, that has a high affinity for LCFA and having a role in facilitating FFA transport across the cell membrane (109). It was also isolated on the apical surface of TRC on the tongue, stomach, intestine and on the surface of macrophages, adipocytes, muscle cells, endothelial cells and platelets. Interesting is also data that CD36 specific inhibitor, sulfo-N-succinimidyl oleic acid ester attenuates its response (108). As well CD36 knock out mice have been generated for which it was shown they lose the ability to distinguish between FFA containing diet over control (107). These mice were able to distinguish a FA solution over gum vesicle, indicating that CD36 is required to distinguish these texturally comparable choices (111). However, role of CD36 in humans is not yet known. Many sequence variations have been identified in human CD36 gene, located on the chromosome 7q11.2 (112), which would be important from genetic point of view, showing that genetic variation in CD36 affects our ability to sense or taste FFA and therefore showing variation in preferences for fatty foods. Thus, examining the relationship between inherited variations of CD36 with fat consumption and oral chemosensory response to fat may help identify individuals predisposed to prefer foods higher in dietary fat. Working model for gustatory perception of LCFA in mouse would be: LCFA released from triglycerides (TG) by lingual lipase bind to CD36, which acts as gustatory lipid receptor in TRCs, which triggers increase in intracellular free Ca^{2+}, which causes release of neurotransmitters by TRC. Animal experiments on rats, including three long chain fatty acids (oleic, linoleic, α-linolenic) suggested that stimulation by fatty acids (FAs) in the oral cavity may provide the chemical information underlying selective behavior toward FFA (19). Electrophysiological recordings on FA chemical information was performed on twins. Dripping FAs on the tongue failed to trigger any electrical response from the CT nerve leading from the fungiform papillae distributed on the lower anterior portion of the tongue (113). However, it was reported in rats, that FFA trigger chemical sensitivity in oral cavity and that glossopharyngeal nerve transmit information to the brain (114). It was also shown that chemical reception of fat centers is triggered by FFA and not by the triglycerides, which actually constitute the bulk of fats. One explanation would be that Ebner's gland, lying in the vicinity of the circumvallatae papillae, where gustatory cells including FAT and CD36 are located, are immersed by lingual lipase in their secret that would split triglicerides to FFA before they would reach these points (12).

Besides CD36 receptor also fatty acid transporter (FAT) was found on the apical part of TRCs in the circumvallatae papillae (12). In CD36/FAT null mice it was shown that they do not recognize FFA (113), which suggested that CD36/FAT acts as a sensor

for LCFAs, which are a major form of fat involved in preferable taste. Recently it was proposed that FFAs in dietary fat may be perceived chemically in TRCs as a basic tastant, accepted into CD36/FAT receptor in the circumvallatae papillae on the tongue, which would serve to recognize LCFAs on the tongue and neuropeptidies, such as β-endorphin or dopamine are released in the brain (12). β-endorphin was shown to be released 15 min after fat intake (111) and dopamine in the Nac was released during sham licking of 100% corn oil (12), which clearly and altogether with described studies show that signals of dietary fat are accepted in the oral cavity and transmitted to the brain, and neuropeptides and neurotransmitters, such as β-endorphin and dopamine were released just after fat intake.

Interestingly chronic fat diet is associated with reduced vagal sensitivity in rodents. This desensitization could be due to dynamic regulation of CCK1R in vagal afferent neurons, since the number of receptors decrease rapidly in the response to fat indigestion. Also expression levels of receptors for GLP1 and leptin by vagal neurons seems to be downregulated by lipids as found for CCK1R (110, 115). This dynamic regulation might account for some of the reduced ability for lipids to satiate in comparison to carbohydrates and proteins.

Interestingly also dopaminergic system plays an important role in lipid preference. Pharmacological inhibition of D1R and D2R in rats has shown food increases reference for fatty foods in dose-response manner, which is important because it was shown that feeding with such food increases dopamine levels in Nac, which is a key component of pleasure and reward circuits and this is decreased by antagonists (116).

Conclusion or making sense of taste

Over the years knowledge about taste perception has raised rapidly. Many candidate receptors are already known to be mediating different taste modalities, however exact pathways and their cooperativity in different pathways still remains unknown. By expanding knowledge about receptors and signal transduction mechanism they are eliciting many options are open to use the knowledge in applicative way. One of the options is to precisely control perception of taste by maneuver the peripheral sensory apparatus and its function directly with small molecules, similarly as have been done in an imprecise way by adjusting the flavors of foods. Basically each flavoring ingredient can be regarded as an agonist or perhaps allosteric modulator. By precisely controlling taste sensation on the level of the sensory receptors in the tongue, we might be able to modulate, turn on off or fine tune taste sensations. Blockers of aversive tastes would be appreciated to help improve patient compliance with unpalatable orally administered therapeutics by influencing 7TM receptors or ion channels as targets. Secondly, important is also interface between taste and indigestion, especially in the case of sweet taste, in connection to obesity and diabetes. By defining why some people are more sensitive to fat taste than the others and modulating the perception high, daily intake of fat could be prevented. On the other hand appetite could be increased in the anorexia as a consequence of manifestation of disease. For example not only in human medicine also in veterinary medicine lack of pet's appetite is evidence that pet is suffering. Especially cats are subjected to anorexia. Mechanisms underlying decreased food intake are complex and not completely understood and one segment is regulation of appetite and mechanisms underlying it. By knowing more about taste mechanisms, "mouth feel", extremely significant factor influencing dietary preference in cats could be influenced and preparing more appealing, masked food.

An important aspect of taste research in future should also be performed in the area of taste receptor genes. The important question to be addressed include finding genes that encode a complete repertoire of taste receptors for different taste qualities, as well as genes that encode proteins involved in taste transduction and transmission, taste bud cell turnover and connectivity between TRCs and afferent nerves. Studies of allelic variation of taste receptors would be helpful to elucidate individual differences in taste perception, food choice, nutrition, and health and to understand functional organization of receptor domains and their ligand specificities.

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ZAZNAVANJE OKUSA: ANATOMSKI IN MOLEKULARNI MEHANIZMI

V. Kubale

Povzetek: Zaznavanje okusa igra ključno vlogo pri izbiri hrane in posledično vpliva na način prehranjevanja. Dojemanje okusa nam pomaga izbirati informacije o različnih kemikalijah v okolju. Do sedaj je opisanih pet osnovnih vrst okusa: sladek, kisel, slan, grenek in umami. V zadnjem času se pojavlja vedno več raziskav in člankov o zaznavanju maščobnih kislin v hrani, t.i. maščobnokislinskem okusu, ki morda postaja šesti osnovni okus, obstoj novih kategorij okusa se še vedno raziskuje. Vsak od osnovnih okusov ima različne funkcije. Umami in sladek okus sta kalorična detektorja, ki nam potešita hedonske čute, sposobnost zaznavanja slanega okusa je pomembna za uravnavanje količine natrija v organizmu in tako še posebej pomembna pri rastlinojedih živalih, kisel okus nam pomaga zaznati nezrelo in pokvarjeno hrano, grenak okus pa prispeva k zaznavi toksinov v hrani. Tako bi lahko bili izsledki raziskav na področju zaznave maščobnih kislin čisto logični: če lahko zaznavamo sladko (ogljikove hidrate) in umami (proteine), je smiselno pričakovati, da imamo tudi sposobnost okušanja maščob. Pri vsakem načinu zaznavanja okusa gre za prenos znotrajceličnega signala preko različnih tipov receptorjev, ki so na različnih okušalnih celicah različnih okušalnih brbončic. Obstajajo na različnih regijah jezika in njegove okolice in z draženjem živčnih vlaken sodelujejo pri zaznavi okusa. Različni kanalčki in receptorji, ki vključujejo tudi receptorje s sedmimi transmembranskimi območji (receptorji 7TM) so posamič ali v sodelovanju (npr. kot heterodimeri) vključeni v zaznavo okusa s sprožanjem različnih poti znotrajceličnega signaliziranja istočasno ali pa vsaka s svojim namenom. V preglednem članku so opisani do sedaj poznani okusi, od anatomskih osnov do molekularnih mehanizmov.

Ključne besede: okus; sladko; kislo; grenko; slano; umami; zaznavanje maščob v prehrani; anatomija; receptorji 7TM; kanalčki; prenos signala

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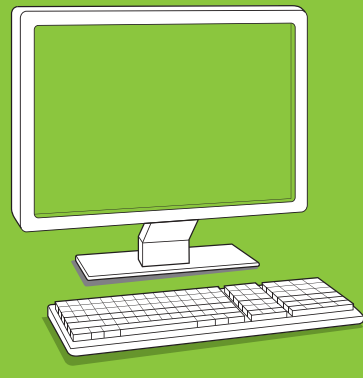

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