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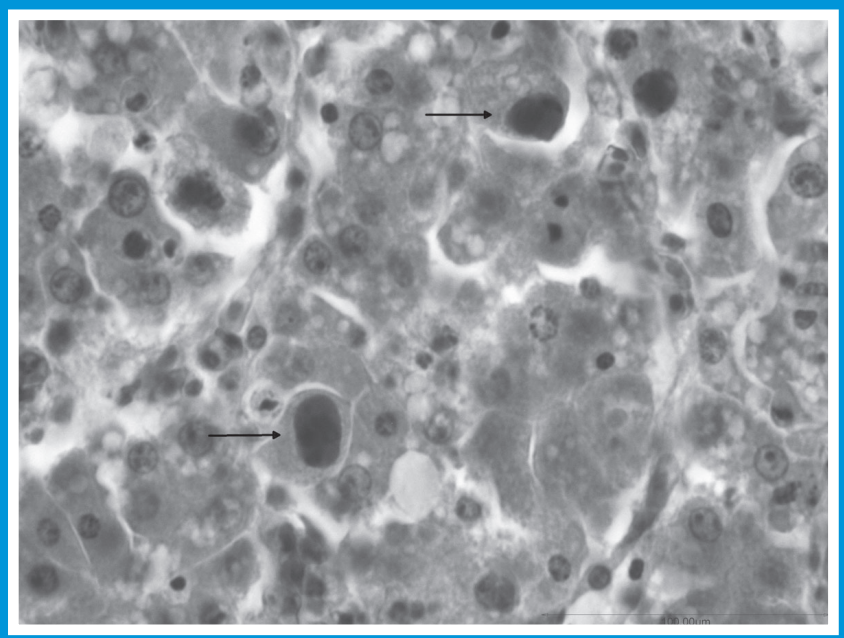
SLOVENSKI VETERINARSKI ZBORNIK



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Review Article

APPETITE REGULATION AND OBESITY: EMPHASIS ON GHRELIN AND GHRELIN RECEPTOR

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Summary: Obesity is one of the leading preventable causes of death worldwide. In its epidemic it is of increasing interest for the pharmaceutical industry to develop drugs that reduce appetite. By reducing appetite overall energy consumption is also reduced. The idea is simple; however, the hormonal system and mechanisms regulating energy intake are extremely complex and therefore drug development is not straightforward. Throughout the central nervous system (CNS), central and peripheral hormones are involved in food intake and body weight balance. They are tightly regulated by the hypothalamus, brainstem and reward circuits, on the basis of both cognitive inputs and diverse humoral and neuronal signals of nutritional status. Several peptides and hormones such as: neuropeptide Y (NPY), melanocortins, cocaine and amphetamine-regulated transcript (CART), peptide YY (PYY), pancreatic polypeptide (PP), cholecystokinin (CCK), oxyntomodulin (OXM), glucagon-like peptide 1 (GLP-1), glucose-dependant insulinotropic hormone (GIP), bombesin, leptin and one of the latest discovered and not yet well known - ghrelin, have all revealed an important role in short- and long-term regulation of food intake. This review summarizes the complexity of factors involved in the regulation of appetite and food intake in different areas of the brain, especially in the hypothalamus, and the relationships between the central and peripheral peptides and hormones involved, with emphasis on ghrelin and its receptor together with their potential role as targets for treatment of obesity. The ghrelin receptor originally received considerable attention from pharmaceutical companies because of its prominent role in the release of growth hormone. However, the discovery of the orexigenic properties of ghrelin turned the ghrelin receptor (ghrR) into a target for anti-obesity drugs.

Key words: appetite regulation; obesity; ghrelin; ghrelin receptor

Introduction

Obesity is one of the most prevalent nutritional problems worldwide; therefore it is important to understand the role of the brain, the peptides and hormones secreted by brain, as well as the periphery organs that regulate appetite and food intake. Two-thirds of North Americans are overweight or obese (1), indicated by a body mass index (BMI) exceeding 30 kg/m^2. Similar trends are observed in Slovenia and other parts of Europe. Being overweight, especially if the body fat is concentrated around the abdomen, is associated with higher risk of various diseases: type 2 diabetes, hypertension, ischaemic heart disease, stroke, endometrial carcinoma, osteoarthritis, gall stones and cancers (colon, kidney, endometrial and postmenopausal breast cancer) (2). Even obesity in pets, especially dogs and cats, is becoming more frequent. Proportions of overweight and obese dogs in the USA range from 23% to 41%, with about 5.1 % classed as obese. In Australia the proportion of obesity in dogs and cats is even higher (7.6 % and 6.4 %, respectively) (3). Furthermore it has been shown that the risk of obesity in dogs is related to obesity of their owners; however, no correlation between cats and their owners was observed (4).

With the expanding knowledge about the areas of the CNS and the central and peripheral hor-

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mones involved in food intake and body weight balance, we are increasing our ability to influence the processes. Hormones, and more so their receptors, represent potential targets in the development of novel anti-obesity drugs. The neuroscience of obesity and the connecting knowledge about all factors influencing obesity is a target of intense interest. The emphasis of research is focused on transferring the knowledge into new treatments for obesity and its related diseases (5). Recent areas of interest are the orexigenic (appetite stimulant) properties of ghrelin and its receptor - ghrelin receptor (ghrR) (6). Discovery of both turned ghrR into a target for pharmaceutical industry to develop anti-obesity drugs.

Peripheral and central appetite regulation

1. Central nervous system (CNS)

The most important part of the brain that processes signals from periphery regarding nutritional status is the hypothalamus, especially the morphologically defined areas such as the lateral hypothalamic area (LHA), ventromedial hypothalamus (VMH), arcuate nucleus (ARC), paraventricular hypothalamus (PVH), perifornical area (PFA) and dorsomedial hypothalamus (DMH). Besides hypothalamus, nucleus tractus solitarii (NTS) also plays an important role in appetite regulation.

LHA was defined as the hunger promoting feeding centre that includes neurons that produce orexigenic peptides. VMH was defined as the satiety centre, which when stimulated suppresses the desire for food. Lesions in this area have been shown to elicit rapid excessive food ingestion (hyperphagia) and abnormal body weight (7). It secretes specific anorexigenic and orexigenic neuropeptides and expresses high levels of VMH brain-derived neurotrophic factor (BDNF). VMH lesion-type hyperphagia and obesity could also be accomplished by other defects (8).

The ARC is one of the highly important areas for receiving signals from the periphery regarding appetite control in the CNS. It is closely connected to the median eminence (ME), which is not entirely protected by the blood-brain barrier (9), therefore enabling ARC to be accessible for satiety or starvation circulating signals of energy balance, such as insulin and leptin. The main neurotrasmitters in the ARC are pro-opiomelanocortin (POMC), yielding the melanocortin MSH as a cleavage product and cocaine and amphetamine-regulated transcript (CART), which both inhibit food intake (10). Furthermore, it was shown that PVH is very important for feeding behavior. Lesions in PVH cause hyperphagia in rats (7) and if an orexigenic signaling molecule (NPY, GAL, orexins, GABA, opioids, norepinephrine and epinephrine) is microinjected into PVH, it stimulates food intake (8). In feeding, it is involved in the opposite manner to LHA. PFA is one of the most sensitive areas for NPY stimulation of feeding. DMH has a role in the modulation of food intake. Lesion studies of this region resulted in hyperphagia and obesity, although not to the same degree as lesions in the VMN (8). Reward pathways that involve complex interaction between several signaling systems such as the dopaminergic and endocannabinoid system are also important. Food intake stimulated by central and peripheral administration of endocannabinoids is believed to be mediated through the cannabinoid receptor type 1 (CB1), which co-localizes with CART, MCH and orexin peptides in the hypothalamus. Opioids are important in the reward circuitry, as mice lacking either β-endorphin or enkephalin do not respond to the reinforcing property of food regardless of palatability (10).

On the other hand, the NTS as the visceral sensory part of the brainstem receives sensory inputs from the larynx, intestinal and respiratory tracts, heart, large blood vessels and taste buds. The NTS and the reward pathways mainly in nucleus accumbens (NAc) are involved in the control of energy intake. The NTS is in close contact with the area postrema (AP) which has an incomplete blood brain barrier. Therefore NTS is like the ARC, able to respond to peripheral circulating signals as well as receiving vagal afferents signals from the gastrointestinal (GI) tract and afferent signals from the glossopharyngeal nerves. Several connections between the hypothalamus and the NTS exist, including the glucagon-like peptide 1 (GLP-1) neuronal circuit, which is believed to be of major importance in the signaling circuit of the brainstem (11).

2. Neuropeptides in CNS

Various neuropeptides are involved in mediating signals regarding energy balance, such as neuropeptide Y (NPY), melanocortins, as well as cocaine and amphetamine-regulated transcript (CART). A review of examples is presented in Table 1.

Table 1: Overview of neuropeptides in the CNS involved in mediating signals regarding the energy balance

	Location	Neuropeptide	Effect (neuropeptide)	Receptor	Effect (receptor)	Reference
NPY	- ARC - autonomic nervous system	- 36 amino acids with many tyrosine residues - structurally related to PP and PYY	- regulation of energy balance - increases food intake and proportion of energy stored as fat - decreases physical activity - role in memory and learning - epilepsy - anorexia nervosa	- 7TM receptors: NPY_{1-5} (brain), NPY_6 (orexigenic) - $G\alpha_i$ coupled - combined signaling through these receptors and other unknown receptors	- role in eating disorders (obesity)	(10, 12-17)
Melanocortins	- ARC - NTS	- peptide hormones - cleavage products of proopiomelanocortin (POMC) in the pituitary gland - different forms of melanocyte-stimulating hormone (MSH) - adrenocorticotropic hormone (ACTH)	- suppresses food intake - POMC gene or gene product mutation or abnormally processing - early-onset obesity	- melanocortin receptors MC1R - MC5R in hypothalamus - very important MC3R and MC4R - $G\alpha_s$ coupled	- MC4R role in regulation of food intake and body weight - 3-5 % of the cases of high obesity in the humans are caused by MC4R mutations - early-onset obesity in humans with deletion or blockade of MC4R	(10, 18, 19)
CART	- hypothalamus - pituitary endocrine cells - adrenomedullary cells - somatostatin cells - rat antral gastrin cells	- anorexigenic peptide - neurotransmitter	- roles in reward, feeding, stress - endogenous psychostimulant - regulates energy homeostasis and interacts with several central appetite circuits	- not yet identified - *in vitro* studies show that CART binds to a specific 7TM receptor coupled to $G\alpha_i/G_o$	/	(8, 11, 20-23)

3. Peripheral Signals

Signals from the periphery importantly influence the energy status of the body, as well as the amount of fat and glucose in the blood. These signals are hormones secreted from various organs in connection with meal initiation satiety and long-term energy changes. The most important are peptide YY (PYY), pancreatic polypeptide (PP), cholecystokinin (CCK), oxyntomodulin (OXM), GLP-1, gastric inhibitory polypeptide (GIP), bombesin, leptin, adiponectin, resistin, visfatin and ghrelin. Review of examples is presented in Table 2. Ghrelin and its receptor are described in more detail below Table 2.

Table 2: Overview of neuropeptides in PNS that influence the energy status of the body, amount of fat and amount of blood glucose

	Location and secretion	Neuropeptide	Effect (neuropeptide)	Receptor	Effect (receptor)	Reference
PYY	- esophagus - stomach - duodenum - jejunum - L cells in ileum and colon (with GLP-1) - neurons - brainstem - concentration in the circulation increases after food ingestion and decreases on fasting - obese people secrete less PYY than non-obese people - released in response to feeding (presence of carbohydrates, lipids and proteins in the GI tract)	- 36 amino acids two forms: - $PYY_{1-3}6$, PYY_{3-36} structurally related to NPY and PP - it crosses blood brain barrier	- slows gastric emptying - increases efficiency of digestion and nutrient absorption after meal - reduces appetite - weight loss was observed after chronic peripheral administration of the peptide to mice - iv administration of PYY3-36 strongly decreased food intake and weight loss in humans	- 7TM receptors: NPY (especially for PYY_{3-36}) - PYY_{3-36} preferentially binds to NPY_2 and NPY_5 - $G\alpha_i$ coupled	- role in the food intake and immnune response	(24-29)
PP	- PP cells in endocrine pancreas secretion in humans is increased after a protein meal, fasting, exercise, acute hypoglycemia - secretion is decreased by somatostatin and iv application of glucose - the amount of peptide released depends upon calorie intake and the composition of a meal	- 36 amino acids - structurally related to PYY and NPY - does not cross the blood brain barrier	- regulates the endocrine and exocrine pancreas secretion - effects hepatic glycogen levels and GI secretions - peripheral infusion of PP reduces food intake, while central administration increases food intake	- 7TM receptors: NPY1, NPY_2, NPY_4, NPY_5 - $G\alpha_i$ coupled	- could mediate the orexigenic effect	(9, 30, 31)
CCK	- GI tract - enterocrine I cells (jejunum, duodenum) - secreted in response to the presence of nutrients in the lumen of GI tract - peripheral CCK crosses the blood-brain barrier and acts directly in the CNS	- different post-translational modification of the *CCK* gene product, preprocholecystokinin - e.g. CCK8, CCK33 and CCK58	- satiety hormones - stimulate digestion of fat and protein and act as a hunger suppressant - able to detect the presence of fat in the chime and inhibit gastric emptying and gastric acid secretion, together with mediating digestion in the duodenum - stimulatory effects oppose those of ghrelin - effect is dependant on the vagus nerve	- cholecystokinin B receptor (CCK2) - widely distributed in brain areas such as the NTS, AP and DMH - adrenal cortex - sensory fibers of the vagus nerve in the pyloric sphincter - $G\alpha_s$ and $G\alpha_q$ coupled	- inhibit gastric emptying, which might be involved in the satiety effect of CCK	(10, 29, 32, 33)

	Location and secretion	Neuropeptide	Effect (neuropeptide)	Receptor	Effect (receptor)	Reference
OXM	- intestinal L cells of ileum and other parts of intestines - co-secreted with GLP-1 and GLP-2	- 37-amino acids - group of numerous tissue-specific cleavage products of proglucagon	- satiety hormone - appetite reducing effects when administered centrally or peripherally - could be used as a weight loss treatment - reduces circulating ghrelin levels in humans and rodents by 44 % and 20 %	- family B 7TM GLP-1 receptor glucagon receptor - $G\alpha_s$ coupled - cAMP accumulation with almost the same potency as glucagons	- L cells in ileum and colon (with GLP-1) - very important in appetite regulation and energy homeostasis - one of the most interesting new targets in the management of type 2 diabetes and obesity	(34-38)
GLP-1	- intestinal L cells - ileum and other parts of intestine (together with PYY and OXM) - secretion is dependent on the presence of nutrients in the lumen of the small intestine by carbohydrates, proteins and lipids - an inhibition of NPY signaling by GLP-1 and an increase by exendin is observed, indicating that the GLP-1 signal is mediated via NPY neurons	- tissue-specific cleavage product of proglucagon - biologically active forms of GLP-1 are: GLP-1-(7-37) and GLP-1-(7-36)	- satiety hormone - antihyperglycemic hormone - inhibits pancreatic β-cell apoptosis - stimulates the proliferation and differentiation of insulin-secreting β-cells - inhibits gastric secretion and motility - decreases acute food intake when administered centrally or peripherally to rats	- family B 7TM - GLP-1, GLP-2, GLP-3 receptors - central administration of exendin, a GLP-1 receptor antagonist abolishes anorectic effect of GLP-1 in OXM - OXM and glucagon are biased ligands on the GLP-1 receptor - $G_{\alpha}s$ coupled	- very important in appetite regulation and energy homeostasis - one of the most interesting new targets in the management of type 2 diabetes and obesity	(34, 39-42)
GIP	- synthesized and secreted after ingestion of fat from the K cells in the intestines - mucosa of duodenum and jejunum of the GI tract - transported by blood	- secretin family of hormones	- glucose-dependant insulinotropic hormone - increases secretion of insulin before rise in blood glucose is observed - effect on adipocytes, enhancing fatty acid synthesis and their incorporation into triglycerides - in ruminants role in nutrient partitioning in milk production (lipid metabolism)	- 7TM receptors - GIP receptors - on β-cells in the pancreas - $G\alpha_s$ coupled	- effect on glucocorticoid metabolism	(43, 44)
bombesin	- most likely secreted from the intestines	- 14-amino acids - closest homologs: neuromedin B (NMB) and gastrin releasing peptide (GRP)	- stimulates gastrin release from G cells - causes satiety with markedly increased plasma levels after feeding - together with CCK, is the source of negative feedback signals that stops eating behavior - peripheral and central administration of bombesin reduces food intake	- 7TM receptors - bombesin receptors: BB1, BB2 and BB3 - BB3 is of great interest for pharmacological industry as a drug target - $G\alpha_q$ coupled	- regulation of endocrine processes and metabolism responsible for energy balance and adiposity	(12, 41, 45, 46)

	Location and secretion	Neuropeptide	Effect (neuropeptide)	Receptor	Effect (receptor)	Reference
leptin	- fat tissue - secretion is proportional to the amount of white adipose tissue in the body	- 16 kDa - a product of the *ob* (obesity) gene, located on chromosome 7 in humans	- key role in regulating energy intake and its influence on appetite and metabolism - signal for status of energy stores - reduces appetite in response to feeding - obese people develop resistance to leptin - suppresses NPY expression in the brain - chronic administration - reduction in food intake and body weight - deficiency of the hormone has shown the opposite effect	- leptin receptor - LEP-R; CD295 (cluster of differentiation 295) - a protein in humans encoded by the *LEPR* gene	- the absence of a leptin or LEP-R - uncontrolled food intake - severe obese phenotype - variations in the leptin receptor have been associated with obesity	(47-52)
adiponectin	- fat tissue - releases into blood and abundant in plasma - levels are inversely correlated with body fat percentage in adults - plasma concentration of adiponectin is suppressed in obesity and when insulin levels rises	- 244-amino-acids	- modulates metabolic processes, including glucose regulation and fatty acid catabolism - role in the suppression of the metabolic disorders that may result in type 2 diabetes, obesity, atherosclerosis - non-alcoholic fatty liver disease (NAFLD) and is an independent risk factor for metabolic syndrome - contributes to increased energy expenditure by activating AMP-activated protein kinase in liver and muscle, leading to an increase in glucose utilization and fatty-acid oxidation in these tissues	- adiponectin receptors in the skeletal muscle (AdipoR1) and liver (AdipoR2)	- reductions in adiponectin receptors may play roles in the development of insulin resistance, type 2 diabetes, metabolic syndrome, and cardiovascular diseases that are linked to obesity	(53-57)
resistin	- adipose tissue of mice and rats, as well as macrophages of primates, pigs and dogs - activated by specific cytokines - a linkage to a certain type of inflammation connected with development of insulin resistance	- cysteine-rich protein - cytokine	- involvement with obesity and type 2 diabetes - may contribute to the mechanism of obesity-induced insulin resistance	- resistin receptor	- role in insulin resistance and type 2 diabetes	(58)
visfatin	- adipose tissue	- adipokine - cytokine	- promotes B cell maturation - inhibits neutrophil apoptosis - acts as an insulin mimetic by binding t insulin receptors and thereby stimulating glucose uptake	- might bind to insulin receptor	/	(59)

	Location and secretion	Neuropeptide	Effect (neuropeptide)	Receptor	Effect (receptor)	Reference
ghrelin	- X/A like cells in the oxyntic glands of the gastric fundus mucosa in the pre-meal situation (plasma levels decrease 80% after removal of stomach) - small intestine - kidney - immune system - placenta - gonads - pituitary - adrenal cortex - lung - hypothalamus - pancreas - collocalize with glucagons in X cells of pancreas or beta cells of pancreas or in E cells of pancreas	- 28 amino acids - chromoseome 3p - 3 introns - encodes 511 base pair cDNA	- only peripheral hormone that stimulates food intake - promotes adiposity if administered peripherally - effect on secretion of growth hormone	- 7TM receptor: ghrelin receptor (ghrR) - NPY/AGRP neuron in ARC - $G\alpha_q$ coupled	- mRNA found in hypothalamus, pituitary gland, pancreas, adrenal gland, spleen, myocardium, vagal nerve	(60-69)

Ghrelin and ghrelin receptor (ghrR)

The gastric hormone ghrelin was identified as the endogenous ligand for the former orphan receptor, the growth hormone secretagoue receptor (GHS-R_{1a}), later named ghrelin receptor (ghrR) (6). Pairing of the hormone to the receptor 20 years after its cloning made the discovery of ghrelin an example of reverse pharmacology. Both names for the receptor are still in use; however, in November 2005, the name "ghrelin receptor (ghrR)" was officially established by the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) (70).

Different natural isoforms of ghrelin exist. One of them is isoform des-Gln 14-ghrelin, which has the same activity as ghrelin; however, it is present in much lower amounts in the serum (71). Interestingly, it was shown that the entire sequence of ghrelin is not necessary to exert its activity, therefore this isoform and other short peptides are very important. Short peptides encompassing the first four or five residues of ghrelin were capable of activating ghrR at about the same efficiency as the full length ghrelin in calcium mobilization assays *in vitro*. 80-90% of circulating ghrelin is in the non-acylated form which is not able to bind to and activate ghrR (6).

Ghrelin is able to reach the brain through the areas where the blood brain barrier is incomplete. A minor quantity of ghrelin is produced within the hypothalamus in neurons adjacent to the third ventricle and between the VMH, DMH, PVH and ARC, therefore ghrelin modulates the neurotransmission and interacts pre- and post-synaptically with NPY/AGRP, POMC and CRH circuits (72). Ghrelin transduces signals to hypothalamic regulatory nuclei that control energy homeostasis. NPY/AGRP neurons of the ARC are major targets for ghrelin. In the hypothalamus, ghrelin increases the firing rate and induces increased expression and release of NPY and AGRP. Ghrelin also seems to exert important actions on energy expenditure, both through ghrR on adipocytes and through modulation of thermogenesis. Therefore it has been suggested that the high constitutive signaling activity of the ghrR could serve as a signaling set-point in the control of appetite and energy expenditure, where it would counteract a large number of inhibitory hormones and neurotransmitters such as leptin and insulin (73, 74). Besides its actions through the hormonal route, ghrelin is also believed to exert its actions through the vagus nerve. Ghrelin produced in the stomach could conduct some of its orexigenic signals via the vagus nerve which signals to areas of the brain very much involved in appetite regulation. It is interesting to note that the stomach may play an important role not only in digestion, but also in pituitary growth hormone release and central feeding regulation (75).

The main effect of ghrelin is observed regarding food intake and metabolism. Ghrelin secretion is

regulated by many factors. In humans, ghrelin level changes during the day. High level is detected during fasting, with a peak before meal initiation, and then it drops after food intake (74). In humans the rise in ghrelin is also associated with feeling hungry (76). Ghrelin levels decline to basal within an hour after food intake (74), proportional to the load of ingested calories (77). In rats, ghrelin is released in a pulsatile manner with a regularity of about 2 episodes per hour. Ghrelin levels change in response to acute changes in nutritional status but chronic changes also have an influence on the plasma ghrelin level. Fasting plasma ghrelin levels are lower in obese research participants compared to normal weight participants, while those with low BMI (patients suffering from anorexia nervosa or carchexia) have an increased ghrelin secretion (76). Ghrelin level is decreased by oral glucose load, after secretion and/or expression of OXM, PYY^{3-36}, administration of somatostatin and its natural analogue cortistatin and agonist/antagonist binding of cholinergic muscarinic receptors (67). Levels of ghrelin are increased by energy restriction, thyroid hormones, testosterone, parasympathetic activity, leptin, and low BMI. The importance of ghrelin in the metabolism has not been entirely clarified. Despite all important actions, deletions of ghrelin or ghrR do not have any major effect on food intake and body weight (8).

As reported in several publications on ghrelin and ghrR, ghrelin plays an important role in appetite stimulation and increase of food intake. Chronic administration of ghrelin to lean rats is followed by an increase in food intake and bodyweight (78); however, the increase in fat mass following ghrelin administration is not caused only by increased food intake. When administering ghrelin twice a day to rodents in a period of four days, it was observed that body weight increases due to reduced fat utilization and fat deposition becomes independent of food intake, suggesting a role for ghrelin in lipid metabolism (79). Therefore, ghrelin may stimulate food intake and at the same time induce adiposity by reducing the use of fat as an energy source (79), which was also supported by calorimetric studies. In humans it was shown, that iv administration of ghrelin increases appetite and food intake in normal weight volunteers (80) and increases food intake in patients with cancer-related anorexia by more than 30% (81, 82).

Besides the effect of ghrelin administered either orally or iv, growth hormone (GH) -releasing effect of iv administered ghrelin in pharmacological doses is also described in humans and animals. Co-administration of ghrelin and GHRH has a significant synergistic effect on GH secretion, indicating that they act via different mechanisms (83). The GH-releasing effect of ghrelin varies with age. It increases at puberty. The rise in estrogens at this time leads to an increase in the expression of the ghrR, which is probably responsible for the GH increment. When reaching adulthood, the level reaches a plateau and declines during further aging (67). In both humans and animals, ghrelin was reported to act as a functional antagonist to the GHRH hormone somatostatin (67). In families with naturally occurring ghrR mutation (Ala204Glu), it leads to selective loss of constitutive activity of the ghrR, but does not affect ghrelin affinity, potency, or efficacy to ghrR. Furthermore, a tendency for developing obesity around the time of puberty was observed (84).

Besides appetite stimulating effect, some other effects of ghrelin have also been observed. It was shown that ghrelin stimulates the release of ACTH and prolactin and consequently increases cortisol levels in humans (85). It has also been reported to cause anxiogenic behavior in humans, creating a possible link between the main place of ghrelin production- the stomach and the brain (stress/anxiety). Furthermore, sleeping patterns have been reported to be affected by ghrelin treatment. In rats it improved memory retention (76). Chronic administration of a ghrelin mimic to old mice restored IGF-I levels and stimulated growth and differentiation of the thymus with an increase in the production of T-cells (86). The GI functions of ghrelin in rats are reported to be a slight increase in acid secretion, ileal peristalsis and modulation of gastric motility (76). Ghrelin also has an influence on the cardiovascular system; improving cardiac contractility and performance in humans following ghrelin injection and counter inflammation in these tissues (67). Decreased ghrelin levels are independently associated with type 2 diabetes mellitus, insulin sensitivity and secretion in humans (87), except in lean humans with type 2 diabetes mellitus.

It is interesting to note that normally, a reciprocal relationship exists between leptin and ghrelin levels. It is suggested that leptin plays a regulatory role in the secretion of ghrelin (88), by having a role in the circadian and ultradian rhythmic fluctuation of ghrelin secretion. This makes it tempting to believe that there might be a feedback mechanism between ghrelin and leptin such that ghrelin also has an ef-

fect on leptin secretion. However, according to experiments performed by Sun et al., which included ghrelin knockout and ghrR knockout mice, this is not the case (89). No difference in the postprandial leptin levels was observed, indicating that ghrelin does not effect leptin secretion. Considerable evidence points to leptin being a regulator of ghrelin levels and an important part of the hypothalamic appetite regulating circuit. In March 2010, researchers reported that mice with type 1 diabetes treated with leptin alone or in conjunction with insulin, had better values of blood sugar and cholesterol than mice with type 1diabetes treated with insulin alone, raising the prospect of a new treatment for diabetes (52).

Ghrelin transmits its signal through the ghrelin receptor (ghrR) (40). GhrR has features characteristic of family A 7TM receptors, including conserved cysteine residues in the top of TM-III and in extracellular loop 2, conserved prolines, E/DRY motif, polar transmembrane residues and several potential sites for posttranslational modifications (N-linked glycosylation and phosphorylation) (90). GhrR signals through $G\alpha_{q/11}$, which results in accumulation of inositol (1,4,5)-triphosphate (IP) and Ca^{2+} release. Ghrelin has also been shown to activate the MAP kinase cascade and the PI3-K/AKT pathway (91). GhrR pharmacology started with the synthesis of analogs long before the discovery of its natural ligand (6, 92). It belongs to a subfamily of receptors for peptide hormones and neuropeptides. Besides ghrelin, the family includes receptors for motilin (previously orphan receptor GPR38), neurotensin, neuromedin U (NMU) and orphan receptor GPR39 (40). GhrR is encoded by a single gene found at chromosomal location 3q26.2. As a result of alternate processing of pre-mRNA, two different variants of the ghrR exist (93). The full length ghrR contains 366 amino acids (ghR R1a). The other splice variant, designated ghR R1b, consists of 289 amino acids and has 5 TM regions. Unlike ghR R1a, GhR R1b is not activated by ghrelin or a synthetic analogue such as the ghrR agonist hexarelin or non-peptidyl GHS such as MK-0677 (94).

Importantly it was discovered (84) that the ghrR signals with ~ 50% of its maximal activity in the absence of its ligand. GhrR has constitutive activity by demonstrating a gene dose dependent but ligand independent increase in IP accumulation (85). It was suggested that the constitutive activity of the ghrR could function as an appetite set-point against the signals from the many anorexigenic hormones such as leptin and insulin. The level of the receptor could then be regulated by ghrelin and perhaps by an endogenous inverse agonist, which would act by decreasing the constitutive activity of the receptor. Biological effects different from those seen after ghrelin treatment have been observed following administration with non-acylated ghrelin and synthetic homologs, suggesting the existence of one or more receptors in the ghrelin receptor family that have not yet been identified (60).

Conclusions

The discovery that ghrelin is one of the most powerful orexigenic and adipogenic agents known in mammalian physiology, triggered the exploitation of ghrR antagonists and/or inverse agonists that can be used to treat obesity (60). GhrR antagonist could be used to block the stimulation generated by ghrelin and thereby reduce meal size. A specific inverse agonist of ghrR would lower constitutive activity of the ghrR and thereby lower the set point of signaling from the receptor between meals. This could increase the sensitivity to the multiple inhibitory signals, e.g. leptin, insulin and PYY_{3-36} and consequently eliminate between-meal food intake. The advantages of combining ghrR antagonist and a ghrR inverse agonist in an anti-obesity drug would be that the antagonist could block the effect of the increase in plasma ghrelin seen before meals (40).

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UREJANJE APETITA IN DEBELOST: POUDAREK NA GRELINU IN GRELINSKEM RECEPTORJU

V. Kubale

Povzetek: Debelost je eden od prevladujočih vzrokov smrtnosti po svetu, kljub temu da bi jo lahko preventivno zmanjšali. Epidemska razsežnost debelosti je postala podlaga za veliko zanimanje farmacevtske industrije za razvoj zdravila, ki bi vplivalo na zmanjšanje apetita. Z zmanjšanjem apetita bi se zmanjšal tudi skupen vnos energije. Ideja je enostavna, vendar so hormonski sistem in mehanizmi, ki urejajo vnos energij,e zelo kompleksni in ne omogočajo enostavnega razvoaj takšnega zdravila. V centralnem živčnem sistemu so peptidi in hormoni, ki se izločajo na periferiji in v centralnem živčnem sistemu, vključeni v vnos hrane in urejanje telesne teže. Vsi so tesno povezani. Uravnavajo jih hipotalamus, deli možganskega debla ter sistem nagrajevanja na osnovi kognitivnih zaznavanj in različnih sporočil telesnih tekočin ter živčevja glede prehrambenega položaja. Veliko nevropeptidov, kot so nevropeptid Y (NPY), melanokortini, prepis, uravnavan s kokainom in amfetaminom (CART), peptid YY (PYY), pankreasni polipeptid (PP), holecistokinin (CCK), oksintomodulin (OXM), glukagonu podobni peptid 1 (GLP-1), od glukoze odvisni inzulinotropni hormon (GIP), bombezin, leptin ter eden zadnjih odkritih in zato manj poznanih - grelin, imajo pomembno vlogo pri kratkoročnem in dolgoročnem urejanju vnosa hrane. Pregledni članek povzema zapletene dejavnike, vključene v urejanje apetita in prehranjevanja, od različnih področij možganov, še posebej hipotalamusa, do povezave med ključnimi centralnimi in perifernimi peptidi in hormoni, s poudarkom na grelinu in receptorju za grelin, skupaj z njihovimi potencialnimi pomeni kot tarče za zdravljenje debelosti. Receptor za grelin je sprva pritegnil pozornost farmacevtske industrije zaradi svoje pomembne vloge pri sproščanju rastnega hormona. Njegova apetit spodbujajoča funkcija je spremenila grelinski receptor (ghrR) v tarčo farmacevtske industrije za razvoj zdravila proti debelosti.

Ključne besede: urejanje apetita; debelost; grelin; receptor za grelin

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Original Scientific Article

IMPACT OF VEROTOXIC *E. COLI* O157 IN ANIMALS ON THE HEALTH OF SLOVENIAN HUMAN POPULATION

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Summary: Infection with verotoxic *Escherichia coli* (VTEC) is an important veterinary public health issue. Ruminants are considered the natural reservoir of VTEC and therefore meat consumption is one of the potential transmission routes. Much effort has been dedicated to monitoring programmes so as to measure the prevalence of VTEC in ruminants in Slovenia. The aim of this study was to statistically analyse data from the national monitoring system by calculating the prevalence of VTEC O157, and to test for association between the season, type of sample, age of cattle, and region with the outcome (chi square, logistic regression). Results were compared to data on human infection with VTEC. Some conclusions for further improvement of surveillance systems were drawn. Animal data were collected between 2006 and 2009 for the purposes of monitoring the prevalence of VTEC O157 in cattle (n=818), sheep (n=320), and meat in cutting plants (n=582), where specific attention was paid to sampling different carcasses/batches. Data regarding human infection with VTEC, collected from 2006 to 2008, included information on age group, gender, season of E. coli notification, and VTEC serotype. Prevalence of VTEC 0157 in cattle was 3.1% (95% CI 1.9% to 4.2%), in sheep 0.9 % (95% CI 0.2% to 2.7%), and in meat 0.2 % (95% CI 0% - 1%). Age of cattle was not significantly associated with positive tests; however, there was a statistically significant seasonal effect across all sample types (p=0.035), as positive test results were found predominantly in autumn. Out of 93 notifications of E. coli in humans, only 15 were confirmed as VTEC (16.1%). Calculated incidence rate of human cases in Slovenia was low (0.2, 0.2 and 0.4 cases per 100 000 population in 2006, 2007 and 2008, respectively), and the most frequently affected group were children. Seasonal patterns for tested animals and human cases were rather similar. In animals, however, VTEC O157 only was tested, whereas the most frequent serotypes isolated from humans were O26, O157, O103 and O111. Evidence shows that this pathogen is harboured by cattle; however, the prevalence in meat is rather low. The precise transmission route in human cases of VTEC in Slovenia remains unclear; and foodborne infection is only one of the options. It may well be that new emerging routes are shifting towards environmental transmission.

Key words: zoonosis; verotoxic *E. coli* (VTEC); ruminants; veterinary public health; Slovenia

Introduction

Verotoxic *E. coli* (VTEC) is a bacterium that produces verotoxins (*Vt*), which damage human intestinal cells, cause diarrhoea and potential life-threatening complications (1). There are two major groups of *Vt,* namely, *Vt1* and *Vt2* (1). Whilst *Vt1* is a homogeneous family of toxins, equivalent to the Shiga toxin of *Shigella dysenteriae, the Vt2* group is deemed to have a much more detrimental potential (1, 2). In addition to production of *Vt,* the bacterium needs to have mechanisms for adhering to and damaging intestinal cells. This is achieved through the AE (attaching/effacing) lesion, encoding different genes (eae genes and genes for Vt), and bacteria possessing these two virulent factors are associated with severe clinical manifestation (2). Both these virulence factors are of crucial importance in laboratory conformation methods.

The surface of *E. coli* is covered by O (somatic) and H (flagellar) antigen profiles, which constitute

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the basis for serotyping. There are more than 200 *E. coli* serotypes (2), and VTEC O157:H7 is in the worst repute of them all. Infection with historically notorious VTEC O157 serotype is strongly associated with severe complication, such as haemorrhagic uraemic syndrome (HUS)(3). HUS is caused by circulating verotoxins, damaging kidney cells, and leading to thrombosis and resulting in acute renal failure (3). Therefore, most national surveillance systems focus only on detecting this particular serotype and making it a most frequently reported one. Non-O157 VTEC serotypes, most commonly associated with severe clinical symptoms, include serotypes O26, O103, O111, O113 (4) and O145 (2).

Though a tremendous amount of knowledge on VTEC has been generated over the past 30 years, infection in humans is still a reality. Therefore, infection with VTEC is considered an emerging zoonosis. In this paper, the specific term of VTEC O157 was examined in animals, whilst in relation to humans the term of VTEC was used for all the types of verotoxin producing *E. coli*.

VTEC epidemiology in cattle and humans

VTEC is a bacterium that is part of normal gastrointestinal flora of ruminants, and thus, these animals may act as a potential reservoir of infection (1, 2, 3, 5, 7, 8, 9, 10). Animals may carry VTEC without any symptoms. There are differences in prevalence and shedding of VTEC. Studies of cattle herds in Scotland showed that the so-called super-shedding animals were responsible for the majority of transmissions (11). Other species considered as possible carriers include sheep and, to a minor extent, goats (10).

It has been reported (12) that an annual prevalence of VTEC in cattle faeces was 4.7 %. However, researchers failed to report age distribution of sampled animals, and sampling was limited to animals of up to 30 months of age. Other researchers (13) have reported a prevalence of 1.2 % in cattle, failing to provide information on how the sampling was conducted. The peak prevalence in cattle during warmer seasons has been described by several researchers studying VTEC (12, 14, 15, 16, 17). It has been reported that more animals had been found positive for VTEC in the warmer seasons (15, 16).

Studies suggest that prevalence in meat is lower than prevalence in animal faeces (18,19). The prevalence in sheep has been reported to be lower than in cattle (12,14); however, sheep may still be an important source of infection, particularly through consumption of raw milk or cheese (4).

In 1983, two human cases in Oregon and Michigan were reported, which were characterized by severe cramps and abdominal pain, where initial watery diarrhoea was followed by gross bloody diarrhoea (20). Diagram 1 outlines the pathogenesis. Diagnosis of VTEC O157:H7 should be considered in any person reporting bloody diarrhoea or HUS (21). Additionally, the incidence is greatest within the most vulnerable groups, as children and the elderly. VTEC infection is known to impair the function of kidneys; this is particularly excruciating for children, who can suffer lifelong consequences.

The immune status is a major factor in the susceptibility to VTEC infection. Children and the elderly are most severely affected, as their immunity is either in development or in decline (2). In addition to these age-related physiological factors, curtailed immunity could be due to the following facts: use of antibiotics in animal production, consumption of processed foods, urbanisation with absence of contact with animals, over-cleaning and use of disinfectants in on a daily basis. An increasing body of evidence suggests the role of acquired immunity as an important factor in preventing VTEC infection in early childhood (2). Moreover, repeated exposure of persons residing in rural environment to these organisms is linked with sub-clinical infections, acting as immunisation against the disease (1). For instance, a rural population, which had been exposed to VTEC, had elevated anti-verotoxin antibodies, as compared to a comparable urban population (2). This suggests that exposure to this bacterium, providing that a person's immune system is capable of effective defence, may have a protecting role.

Additionally, the importance of VTEC as an emerging pathogen should be viewed through costs encumbering the healthcare system. A recently published study (22) suggests that in the USA the estimated cost of foodborne illness-related issues amounts to USD 152 billion per annum in healthcare, at workplaces, in addition to other economic costs. Expenditures related to *E. coli* O157:H7 infection alone (requiring hospitalisation) were estimated at USD 14 838 per case (22). The total cost related to this pathogen was estimated at USD 993 million (with 95 % CI 296-1689).

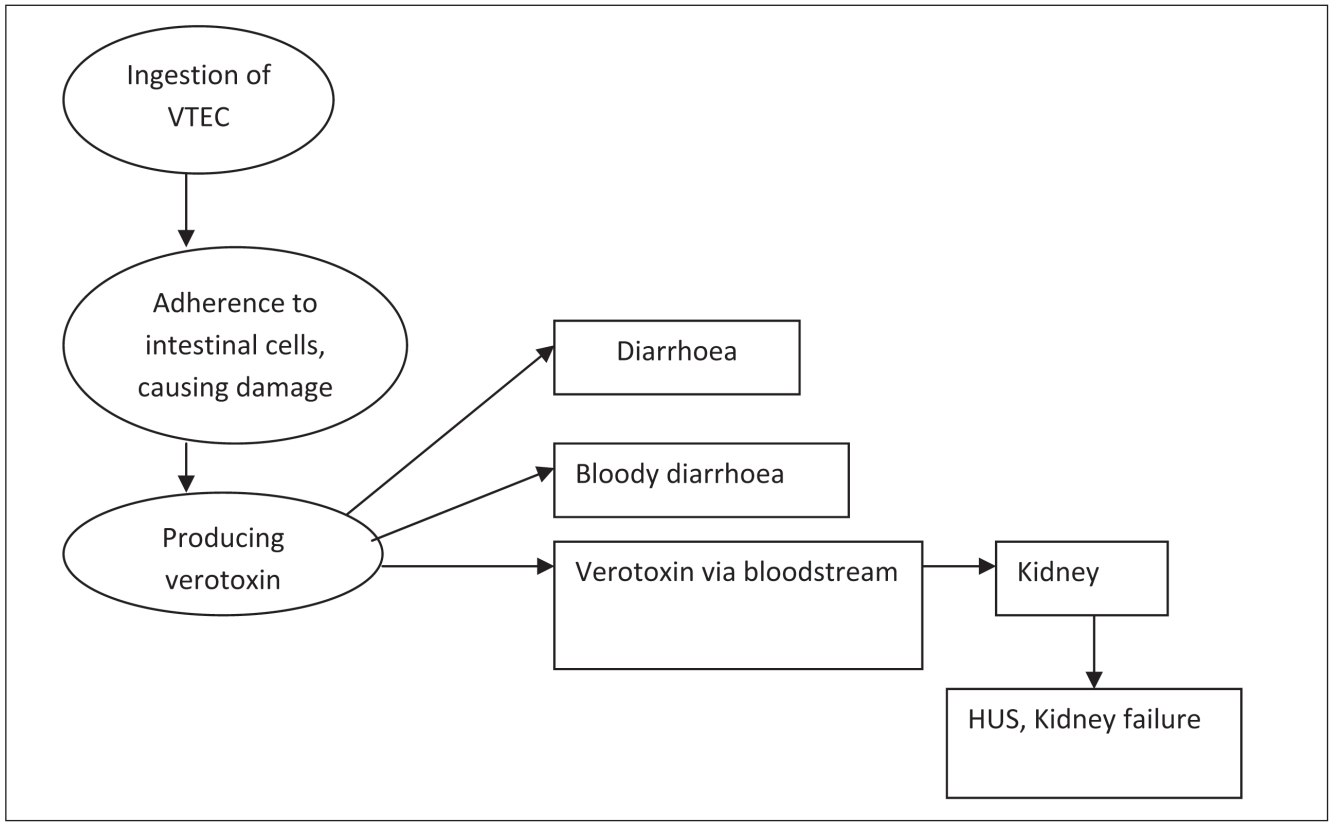

Diagram 1: VTEC infection pathogenesis

Pathways of VTEC infection transmission from animals to humans

Food of animal origin is often found to be the source of transmission to humans (2, 5, 6, 10). A particular threat is posed by undercooked minced meat; grinding of this meat helps to deliver the pathogen into the interior of the meat, where it is more likely to survive cooking (19, 5, 9). The quantity of bacteria required to cause the disease is very low, that is less than 100 cells (10). For a long time, undercooked meat had been considered as the principle source of infection. However, nowadays, knowledge suggests a wider diversity of food types potentially responsible for the spread of infection. These include apple cider and vegetables, such as lettuce, alfalfa sprouts and spinach (5). For example, an outbreak of *E. coli* O145 in USA was reported, where 26 people from multiple states were infected whilst the determinant was contaminated shredded lettuce (23). Cross contamination of vegetables usually happens by water and soil that is contaminated with animal faeces (24). This can be particularly problematic with vegetables, consumed in a raw state. As first, a foodstuff gets contaminated with VTEC (primary source, cross-contamination, or from contaminated workers, processing the produce), and in addition, it is inadequately prepared, stored or cooked, consequently allowing survival of VTEC (4). Pasteurised milk is less commonly reported as primary source of infection; however, since it is used as a raw material for milk products, cross contamination is more likely to occur, especially if the production premises are on the farm (25).

In a 10-year study of outbreaks in the USA (26) researchers report that food contaminated with VTEC was responsible for 61% of the cases. The produce and ground beef were the key sources of infection in 41% of the cases. Scottish researchers (27) found that in 54% of outbreaks caused by VTEC the transmission route was from the environment, while in 40 % it was foodborne. Other Scottish reporters observed that incidence of infection was 1.7- times higher in rural areas, and this factor was over three for children (28). This is supported by the report (29) of outbreaks in Scotland in 2004, where half of the cases were children, and contact with cattle and/or

their excreta was considered the key source of infection.

According to *Trends and sources of zoonoses and zoonotic agents in EU in 2008* (30) top five VTEC serogroups include O157, O26, O103, O145 and O91. Within the EU, children accounted for 64.6% of the 144 HUS cases in 2008, and these were mainly associated with serogroup O157 (30). Infection with VTEC in Slovenia in 2005 and 2006 resulted in two deaths; one elderly man and one child (31). Later there was a fatal case of HUS following infection with O145 VTEC (32). Investigators report that meat and water samples tested positive for *E. coli*, however, not for serogroup O145 (32). They hypothesize that most likely the source of infection could be minced meat, but there were no leftovers to be tested for VTEC.

VTEC surveillance in Slovenia is passive and based on GPs' reporting of *enterohaemorrhagic E. coli* (EHEC). Notification rates are basically the number of patients presented with clinical signs for EHEC infection divided over 100 000 population, but not yet confirmed as VTEC. There is a central laboratory, where final diagnostics is carried out. Only samples from severely affected cases are submitted to central laboratory for the subsequent diagnostic testing for the presence of *Vt*, and thus, only these samples stand for confirmed cases. Incidence rate is the number of cases of VTEC divided over 100 000 population.

VTEC can never be completely eliminated from animal herds, and therefore, enormous efforts have been put into improving the knowledge of effective strategies of decreasing the transmission to humans. The aim of this study was to explore the prevalence and look for associations by statistically analysing the data available from the monitoring programme of VTEC O157 in ruminants in Slovenia.

Material and methods

Data on animals were provided by the Veterinary Administration of the Republic of Slovenia (VARS) and were part of a nationwide study aiming to investigate the baseline prevalence of VTEC O157 in animals and meat intended for human consumption. The surveillance data comprised 1720 records taken from cattle faeces (n=818), sheep faeces (n=320) and cuts of cattle meat (n=582). Sampling took place from January 2006 to December 2009 at slaughterhouses (faecal samples) and at cutting plants (meat cuts) across Slovenia (n=86). These establishments are approved and under official control of VARS. Most of the tested animals (faecal samples) originated from cattle reared within the Republic of Slovenia. Meat cuts sampled in this study were essentially from above mentioned slaughterhouses with a small number of meat cuts from other Member States. The number of samples collected from each slaughterhouse / cutting plant was proportional to its throughput. To prevent clustering of samples from the same herds, samplers were instructed that no more than one sample should be obtained from any one farm of origin on the same day. Moreover, complete records were kept on the origin of sampled animals, and additional attention was given to sampling different batches of meat. Samplers were official veterinarians, who were trained prior to sampling. Sampling of animal faeces included the cutting of a part of the caecum, including the adjacent intestinal wall, and took place immediately after the evisceration. Cuts of meat were sampled from different batches on the premises of cutting plants.

Laboratory diagnostics was carried out by the National Veterinary Institute (NVI), using the ISO 16654:2001 method for detection of VTEC O157. The date of sampling was used for the purposes of analysing the effect of season. Data were subjected to statistical analysis using the statistical software SPSS, Version 18. 0 (SPSS Inc., Chicago, Illinois). Microsoft Office Excel 2003 (Microsoft, United States) was used for calculations of proportions and rates. The variables of interest used from the dataset are the result of testing for VTEC O157, date (season) when sample was taken, age of the animal, type of the sample (cattle faeces/sheep faeces/cattle meat), and location of slaughterhouse by region in Slovenia.

First, different types of samples positive for VTEC O157 were compared with relation to animal age, season and region of sampling. χ^2 test was used to examine association between positive samples and season, and positive samples and region. Logistic regression was used to examine overall association of season with the outcome of testing positive for VTEC. Additionally, logistic regression was used in cattle subset for examination of association with season and age, respectively. Since at least 10 events per variable were required to proceed to logistic regression and vast majority of positive results were found in cattle samples (n=25), the cattle dataset was used to seek for association between age and VTEC. In winter, there were no positive samples

taken from cattle. Where this is not a problem to calculate the odds ratio, it does create problems for logistic regression. Therefore, this season needed to be merged with one of the neighbouring seasons. Hence, winter was merged with spring, both being the colder parts of the year. This was then used as a reference group in logistic regression. The age trend in cattle was examined using categorisation which would allow equally sized groups. Despite the fact that such categorisation offers little practical information, categorisation was extended to ten equally sized age bands.

Ethical approval was granted by the Ethical Committee (Centre for Population Health Sciences, University of Edinburgh). Data of human infections were anonymous and partially obtained from already published sources, and steps to protect the privacy of breeders were taken as well.

Data on human infection with VTEC were obtained from reports on national surveillance of communicable diseases (31, 33) and through personal communication with experts in this field (IPH). Data on human cases were provided for the timeframe 2006-8, whilst notification rates were obtained from published sources and dated since 1997 (31, 33). Notification/incidence rates per 100 000 population were calculated by dividing the number of notifications/cases in each year with midyear census data (34). Specific information on different serotypes of VTEC isolated from patients, age, gender and season distribution of cases were provided by public health professionals from the *Institute of Public Health of the Republic of Slovenia (IPH)*.

Results

Data obtained in slaughterhouses/ meat cutting plants

a) Prevalence of VTEC O157

Data from national monitoring system revealed that there was a statistically significant difference in prevalence of VTEC O157 between types of sample (χ^2 =18.40, d.f.=2, p=0.001). The Table 1 shows that the prevalence was the highest in cattle and lowest in meat. The odds of VTEC O157 outcome were significantly higher in cattle as compared to meat (p=0.004, OR 2.48 to 135.56), but not for sheep relative to meat (p=0.141, OR 0.57 to 53.08).

Table 1: Results on VTEC O157 from different types of samples

Type of sample	N tested	n positive for VTEC O157	Prevalence (%)	95 % CI
cattle	818	25	3.1	1.9% - 4.2%
sheep	320	3	0.9	0.2% - 2.7%
meat	582	1	0.2	0% - 1%
TOTAL	**1720**	**29**	**1.7**	**1.2% - 2.4%**

b) VTEC O157 by season

Table 2 shows that the highest prevalence of VTEC O157 is in autumn (Sep-Nov). There was a statistically significant association between a positive VTEC O157 result and the season (p=0.035), with winter (Dec-Feb) as the reference group. The odds of VTEC O157 were significantly higher in autumn as compared to winter (OR 1.63 to 94.97), but not for spring and summer as compared to winter (Table 2).

Table 2: Association of VTEC 0157 with season of sample (n=1720)

Variable	N samples	n positive VTEC O157	p-value	OR (95 % CI)
Season			0.035	
Dec-Feb	381	1		Reference
Mar-May	490	6	0.152	4.71 (0.57 to 39.30)
Jun-Aug	407	8	0.056	7.62 (0.95 to 61.21)
Sep-Nov	442	14	0.015	12.43 (1.63 to 94.97)
Constant				0.003

c) VTEC O157 by age and region

In sheep, positive animals were aged 3 months, 9 months and one missing age value. The animal, whose meat tested positive, was 24 months of age. The remaining positives were found in the cattle subset, with two missing values.

Sampling took place within 10 VARS Regional Offices (each covering a respective regional unit). Region of sampling was not significantly associated with the outcome (χ^2 =12.67, d.f.=9, p=0.178).

d) VTEC in cattle by season and age

In autumn there was a significant increase in odds of cattle testing positive for VTEC O157 as com-

pared to winter (Table 3). Percentage of cattle testing positive varies nearly 4-fold across age bands, but there is no discernible age trend (Figure 1).

Table 3: Association between VTEC and season of sampling in cattle (n=818)

Variable	N	p-value	OR (95 % CI)
Season		0.021	
Dec-May	408		Reference
Jun-Aug	202	0.119	2.41 (0.80 to 7.25)
Sep-Nov	208	0.005	4.10 (1.52 to 11.09)
Constant			0.015

f) Virulence factors

Vast majority of VTEC O157 strains isolated from animals/meat (n=29) contained key virulence markers, namely, the *eae gene* (93%) for bacterial adherence and *Vt2* (86%), confirmed as being potentially pathogenic to humans (Mičunovič, personal communications).

Data on human infection

a) Notified cases

Between 1997 and 2008 there were 680 notifications of EHEC (30), however, these do not stand for laboratory-confirmed cases of VTEC.

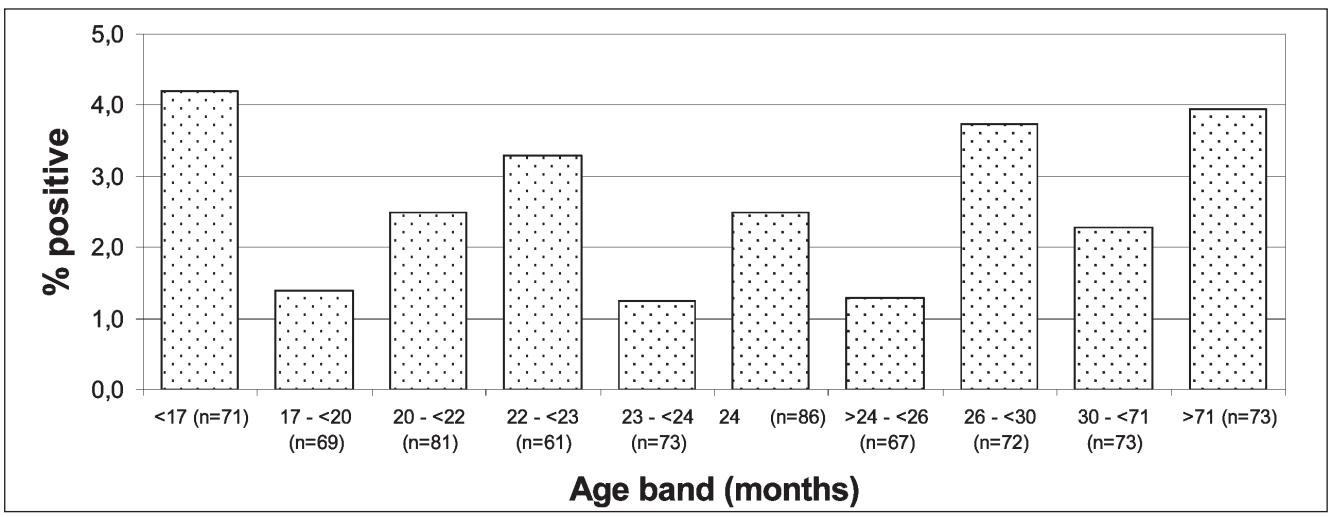

Figure 1: Percentage of cattle testing positive for VTEC O157, (n=726) with n=23 positive and n=2 missing values

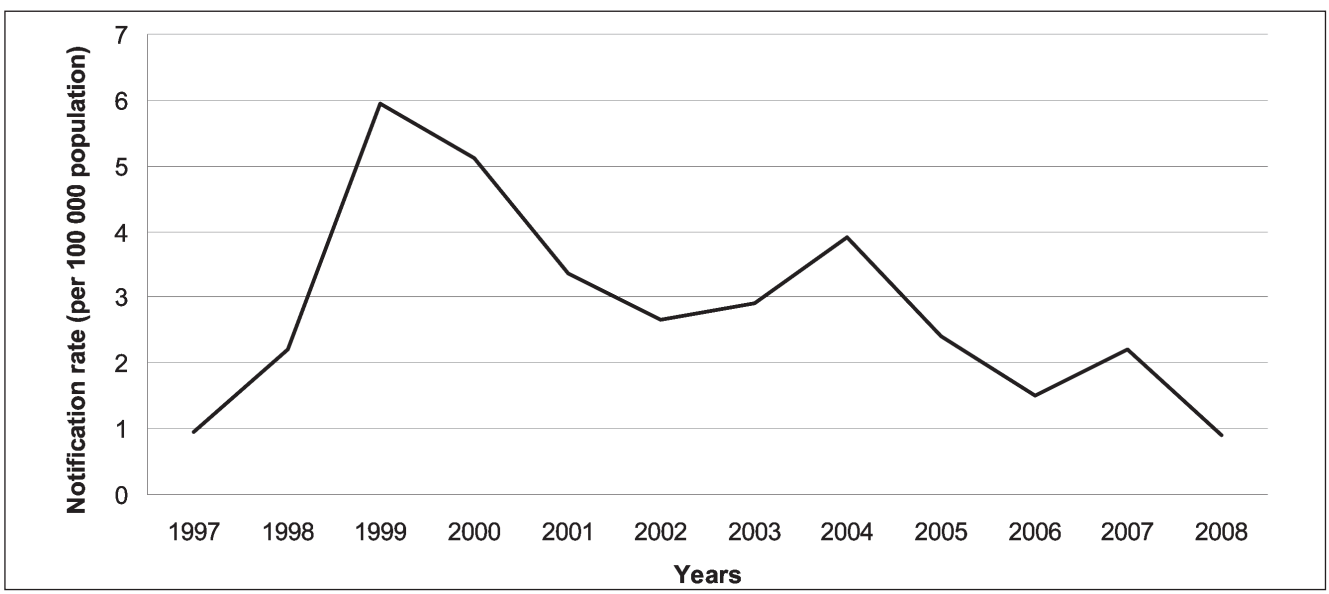

Figure 2: Notification rates of EHEC in Slovenia from 1997 to 2008 (n=680)

b) *Confirmed cases*

Data on patients infected with VTEC are provided from 2006 to 2008, with 93 notifications of EHEC, 15 cases of which were confirmed as VTEC (33, Trkov, personal communications). Majority of cases were children of up to 7 years of age, who comprise 67% of all cases. Most commonly reported gender was male (53%). More than half of the cases occurred in autumn (Sep-Nov) (53%), followed by summer (27%). Incidence rates of VTEC cases per 100 000 population are given in Table 4. Each year, approximately 10% of samples submitted for further confirmation to the laboratory carrying out molecular testing were confirmed positive for VTEC. These isolates have the ability of producing *Vt* or containing relevant genes. Serogroups isolated from patients were O26 (40%), O157 (27%), O103 (20%), O111 (6%), and not defined (6%).

Table 4: Incidence rate for VTEC cases in Slovenia, 2006-8. Data provided with the courtesy of Dr. Trkov from IPH

Incidence rate of VTEC cases per 100 000 population		
2006	2007	2008
0.2	0.2	0.4

Discussion

These findings suggest that the prevalence of VTEC O157 in sampled ruminants in Slovenia is low. Prevalence in cattle is consistent with similar research conducted elsewhere (12, 14) and with reports from the EU Member States, where prevalence in cattle ranges from 0 % to 7.2 % (29). Sheep showed lower prevalence, of 0.9% (95% CI 0.2% to 2.7%), corresponding the results of two larger studies (12,14). Meat sampled from cattle showed extremely low prevalence, of 0.2% (95% CI 0% to 1%), similar to samples from processing plants and retail (18, 19, 28). Similarly, within the EU Member States, prevalence in meat seems lower as compared to faeces, and ranges between 0% and 1.2 % (30).

The presence of VTEC O157 in cattle excreta can be influenced by the age of the animals; calves of up to four months of age seem to be the key source of infection (9). Others (14) argue that more advanced age is protective against testing positive for VTEC O157. Statistical analysis presented above did not reveal any significant association between cattle age and positive outcome.

Climate plays an important role in infection (2). Summer season association with higher prevalence in cattle has been reported (12, 14, 15, 16, 17). The seasonal effect was statistically significant, with increased odds of testing positive in autumn. This effect of season can further be explained by the fact that autumn temperatures in Slovenia (with the exception of September 2007) during the timeframe of this study were above the average (35). On the other hand, winters were among the coldest, which constitutes a possible explanation of a low number of positive results in this season.

Analyses of surveillance data of human infection with VTEC showed a relatively low incidence rate, ranging from 0.2 to 0.4 cases per 100 000 population, depending on the year. Children are most affected from all the groups. Similarly to animals, infection follows a similar seasonal pattern, i.e. an increased rate in autumn. The serogroups isolated from humans (in order of effect) were O26, O157, O103 and O111. Incidence rate is neither a rate nor a proportion; here, it was used to describe the number of new events of diarrhoea in a defined population within a specified period of time. This was a cross-sectional study, where the prevalence in animals was used as a measure of VTEC O157 occurrence. Thus, it is a point at issue, whether the rate was erroneously compared with proportion. Nonetheless, the incidence of enteric disease is rather frequently close to prevalence, as the disease is of short duration.

The data as presented above indicate that, as compared to meat, cattle are more likely to be a source of VTEC O157 infection. Thus, the emerging routes of transmission should be taken into consideration, in particular the contact with animals during farm visits, environment-related exposure (application of manure to the soil), and contaminated vegetables. It has been suggested that exposure to rural environment can have a protecting role (2). Comparably, the incidence rate in Slovenia is rather low, which is possibly due to the fact that a majority of Slovenian population lives in rural areas (36) and is thus more frequently exposed. Further research combining the veterinary and public health expertises will be most advantageous.

Further improvements in monitoring/surveillance systems

Reliable data from surveillance system are necessary for critical assessment. Limitations of this study include lack of available data, which resulted

in unequal time span of animal data and human cases. Data from communicable disease surveillance systems provide information with limited analytical meaning. Animals are only tested for O157 VTEC, which accounts for one of the crucial gaps in zoonoses monitoring. There could be considerable underestimation of the true prevalence of different VTEC serogroups in animals than if the important pathogen were examined from a broader perspective. For further research on sources of infection/transmission of VTEC in Slovenia, animal testing should necessarily be extended to non-O157 VTEC serogroups.

The above findings demonstrate that 67 % of serogroups isolated from humans belonged to non-O157 VTEC. Report of fatal case of HUS following infection with O145 VTEC in Slovenia (32) points towards extending the testing of animals as potential source of infection to this serogroup. The extended testing of animals to non-O157 VTEC serogroup can potentially lead to a better estimate of current state of VTEC in animals and consequently in the food-chain. In the final phase of drafting this Master's thesis it was brought to the authors' attention that the zoonoses monitoring programme that started in the beginning of 2010 already incorporated the testing for the above recommended serogroups. These baseline data on extended serogroups will be exceedingly valuable for the next evaluation, where the impact of animal isolates will be assessed in the light of those found in humans.

Slovenia needs an upgraded surveillance system for identifying the VTEC cases. This may be achieved through comprehensive testing of all patients suspected of infection with VTEC. Here, the availability of laboratory facilities and resources plays a pivotal role. It may well be that persons living near laboratories (in cities) are more likely to be tested and therefore included in the surveillance. It has been specified by the EU that reporting communicable diseases at the EU level should comprise the confirmed cases only, which includes any person meeting clinical and laboratory criteria (37). Another weakness in the Slovenian surveillance system is the non-exhaustive detection of VTEC infection in humans, where milder cases go unreported. In many other countries, HUS has been shown a reliable indicator of VTEC infection in the human population. For the time being, HUS that accompanies the severely affected cases is not a compulsorily notifiable disease within the Slovenian public health system. By establishing a routine HUS surveillance system, the critically affected cases would be identified as well. Capturing these cases could potentially lead to comprehensive and prompt research of underlying sources of infection. Taking into consideration all of the above, the surveillance of communicable diseases in Slovenia needs to be upgraded in order to provide a most comprehensive survey of the current state, thus facilitating a most efficient treatment of gastrointestinal diseases.

Conclusion

Despite a low prevalence it has been found that cattle and sheep in Slovenia do carry VTEC O157. As regards the Slovenian climate, data suggest that the warmer seasons stimulate the presence of VTEC O157 in animals and thus, as a possible consequence, increasing the number of human cases. Hence, specific attention should be paid to human cases during the warmer months of the year, establishing the possible interlinks with animals by thorough investigation.

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VPLIV VEROTOKSIČNE *E. COLI* PRI ŽIVALIH NA ZDRAVJE SLOVENSKEGA PREBIVALSTVA

K. Hladnik Trček

Povzetek: Okužba z verotoksično *Escherichio coli* (VTEC) je pomemben problem v javnem veterinarskem zdravstvu. Prežvekovalci so naravni rezervoar okužbe, kjer je okužba z uživanjem mesa ena od morebitnih poti prenosa. Veliko truda in sredstev je bilo vloženih v programe spremljanja prevalence VTEC pri prežvekovalcih v Sloveniji. Namen te študije je bil podati statistično analizo podatkov, zbranih v okviru nacionalnega sistema monitoringa. V ta namen je bila izračunana prevalenca VTEC O157 in testirane so bile statistične povezave med spremenljivkami (chi kvadrat, logistična regresija), prav tako je bila navedena primerjava z okužbo pri ljudeh. Ugotovitve te analize so pokazale področja, kjer bi se sistem spremljanja lahko izboljšal. Zbrani podatki pri živalih so del nacionalnega sistema spremljanja VTEC O157, kjer so bili v obdobju 2006-09 odvzeti vzorci iztrebkov goveda (n=818) in iztrebkov ovac (n=320) v klavnicah ter mesa goveda (n=582), odvzetega v razsekovalnicah po Sloveniji.

Podatki o okužbi z VTEC pri ljudeh so navedeni za obdobje 2006-08 in vključujejo podatke o starostni skupini in spolu obolelih, sezoni ugotovitve okužbe ter serotipu VTEC. Prevalenca VTEC O157 pri govedu je bila 3,1 % (95 % CI 1,9 % do 4,2 %), ovcah 0,9 % (95 % CI 0,2 % do 2,7 %) in v mesu goveda 0,2 % (95 % CI 0 % do 1 %). Starost goveda ni bila statistično značilno povezana z rezultatom, pozitivnim na prisotnost VTEC O157. Nasprotno pa je bil letni čas statistično značilno povezan z vrsto vzorca (p=0,035), saj je bila večina pozitivnih rezultatov zaznana v jeseni. Od skupno 93 ugotovitev okužbe z E. coli pri ljudeh je bilo le v 15 primerih potrjeno, da gre za VTEC (16,1 %). Izračunana stopnja incidence pri slovenskem prebivalstvu je bila nizka (0,2, 0,2 in 0,4 primerov na 100 000 prebivalcev v letu 2006, 2007 in 2008). Najpogosteje zbolijo otroci. Jesen je tisti letni čas, ko je več rezultatov pozitivnih na prisotnost VTEC, tako pri ljudeh kot tudi pri živalih. Medtem ko so bile živali testirane zgolj na prisotnost serološke skupine O157, so najpogostejše serološke skupine VTEC pri ljudeh O26, O157, O103 in O111. Dokazano je, da govedo nosi povzročitelja v prebavnem traktu, vendar je prevalenca VTEC v mesu nizka. Pot prenosa za okužbo ljudi v Sloveniji ostaja nerazjasnjena, saj je okužba prek živil le ena izmed možnosti prenosa. Nove porajajoče se poti okužbe z VTEC lahko kažejo na prenos povzročitelja iz okolja.

Ključne besede: zoonoza; verotoksična *E. coli* (VTEC); prežvekovalci; javno veterinarsko zdravstvo; Slovenija

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Original Scientific Article

COMPARISON OF DNA EXTRACTION METHODS TO DETECT *SALMONELLA* SPP. IN TAP WATER

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Summary: Bacteria of the genus *Salmonella* cause a global health problem related to contaminated foodstuffs and faecally-polluted water supplies. The aim of our work was to determine the efficiency and rapidness of *Salmonella* detection in spiked tap water samples by comparison of different methods for DNA extraction. Water samples were spiked with different *Salmonella* loads. Four DNA extraction methods were employed: foodproof™ Sample Preparation Kit I (Biotecon Diagnostics) and QIAamp® DNA Stool Mini Kit (Qiagen) for DNA extraction after an overnight enrichment step, and Adiapure® Water DNA Extraction and Purification Kit (Adiagene) and SmartHelix® Complex Samples DNA Extraction Kit (developed recently in our laboratory) for direct DNA extraction. The extracted DNA was subjected to PCR amplification using *Salmonella* genus-specific primer pair. The SmartHelix extraction kit proved to be more efficient than the Adiapure Water kit for direct detection of *Salmonella* DNA in water. The overnight enrichment step improved the detection of *Salmonella* spp. in samples spiked with low bacterial load, however it extended the analysis time. Results suggest that the newly developed SmartHelix extraction kit should be regarded as a considerable choice when rapid detection of pathogens in water samples is aimed for to prevent disease outbreaks and support safe food assurance.

Keywords: DNA extraction; food safety; pathogen detection; PCR; *Salmonella*; water

Introduction

Salmonelloses are one of the most frequent foodborne zoonoses in industrialized countries caused by consumption of contaminated foodstuffs of animal origin (1). They are associated with abdominal cramps, diarrhea, nausea, vomiting and fever, but can occasionally evolve into severe localized infections or potentially fatal systemic sepsis (2). Since bacteria of the genus *Salmonella* are found in the digestive tract of humans and a variety of animals, faecal transmission to water supplies in regions with poor sanitary measures occurs continually. Contaminated drinking and food-processing water represents a significant source of infection for humans in the third world countries, mostly by the human-adapted *Salmonella enterica* subsp. *enterica* serovar Typhi and *Salmonella* serovar Paratyphi causing severe systemic disease (3). On account of easy dissemination by contamination of food and water supplies, bacteria of the genus *Salmonella* are also considered a potential bioterroristic agent belonging to the category B according to the established classification. There have been cases of intentional contamination reported in the past aiming for economical or political destabilization to create panic among civilian population and intimidate the authorities (4-6).

The main requirement for rapid detection of deliberate or non-deliberate contamination is a sensitive surveillance system that links local laboratories and clinicians with public health professionals to enable the recognition of unusual pattern of reported disease cases (5,7). Traditional cultivation-dependent methods based on ISO 6579:2002 (8), which is

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set as the golden standard for detection of *Salmonella* spp. in food and feedstuffs (9,10), require five working days to confirm positive results. Therefore, standardized procedures are not compatible with the demand for rapid assessment of *Salmonella* spp. contamination in water systems. In order to prevent the dissemination of bacteria, more rapid and sensitive molecular techniques, which are based on the detection of microbial nucleic acids, are needed.

The objective of our study was to investigate the efficiency and rapidness of direct DNA extraction method applying a commercially available kit that was recently developed in our laboratory (Institute of Physical Biology, Slovenia). This new method is especially adapted for DNA isolation from cells concentrated on filters. It was compared to other commercial extraction kits with or without the pre-enrichment of bacterial cells. Four methods for DNA extraction were selected according to their applicability for the type of samples and their ability to remove the inhibitors of the subsequent molecular detection that can be found in the field, clinical or alimentary samples. Detection of *Salmonella* spp. in tap water samples was based on microbial DNA extraction followed by the polymerase chain reaction (PCR).

Materials and methods

Bacterial strain for sample preparation

Reference strain of *Salmonella enterica* subsp. *enterica* serovar Enteritidis (CAPM 5439) was used in the present study. The overnight bacterial culture was grown at 37°C in buffered peptone water (BPW) prepared according to Anex B of ISO 6579:2002 (8), diluted in 2-fold series and used as an inoculum for the tap water samples.

Inoculation of water samples

Thirty liters of tap water were collected and further subdivided into 500-mL samples. Four different bacterial dilutions were spiked in five parallels into water samples to obtain 20 samples with *Salmonella* loads of 18, 36, 72 and 144 colony forming units (CFU) per liter, respectively. The same spiking procedure was repeated three times to obtain sample parallels for different DNA extraction procedures, namely two series of 20 samples for direct extraction and one series of 20 samples for the overnight enrichment prior to extraction. CFU load of the bacterial culture was determined using the standard plate count technique (11) on Rambach agar plates (12).

Extraction of microbial DNA from spiked water samples

Bacteria in spiked water samples were concentrated by filtration through 0.45 μm membrane filters (Sartorius, Germany). After filtration, one series of 20 samples was used for the overnight pre-enrichment in 10 mL of BPW at 37°C. One mL of the obtained enrichment culture was used for DNA extraction using foodproof™ Sample Preparation kit I (Biotecon Diagnostics, Germany; abbreviated as FP-e) and one mL for the extraction using QIAamp® DNA Stool Mini kit (Qiagen, Germany; abbreviated as S-e) according to the manufacturers' instructions. Two other series of 20 samples were used for direct extraction from the filter-concentrated bacteria using Adiapure® Water DNA extraction and purification kit (Adiagene, France; abbreviated as AW-d) and by the method developed in our laboratory, namely SmartHelix® Complex Samples DNA extraction kit (Institute of Physical Biology, Slovenia; abbreviated as SH-d), according to the manufacturers' instructions. Direct extraction was performed immediately after sample filtration. The extraction methodology is summarized in Table 1. More information on the SmartHelix technology is available at http://smart-helix.com/.

Molecular detection of Salmonella spp. in water samples

The isolated microbial DNA was subjected to PCR amplification using *Salmonella*-specific primers ST11 and ST15 (13,14). PCR was performed according to the modified protocol as described before (15). Each individual sample in five parallels was PCR amplified in four replicates to obtain 20 results of detection per each of the four extraction methods. Amplified PCR products were separated on agarose gels by electrophoresis, stained with ethidium bromide (10 μg/mL; Invitrogen, USA) and documented using the GeneGenius bio-imaging system (Syngene, UK).

Data analysis

To statistically evaluate the obtained data, the most probable number (MPN) method was coupled

Table 1: Commercially available kits for DNA extraction from water samples employed in the present study

DNA extraction kit	Manufacturer	Abbreviations	Type of analysis	Type of extraction method	Hands-on time (h)
Foodproof™ Sample Preparation kit I	Biotecon Diagnostics	FP-e	Overnight pre-enrichment in BPW prior to extraction	Chemical/thermal cell disruption and DNA purification with glass fiber spin columns	≥12 + 0.5
QIAamp® DNA Stool Mini kit	Qiagen	S-e		Thermal/proteinase K cell disruption and DNA purification with silica spin columns	≥12 + 1
Adiapure® Water DNA extraction and purification kit	Adiagene	AW-d	Direct extraction	Chemical/thermal cell disruption and DNA purification with ultrafiltration columns	1.5
SmartHelix® Complex Samples DNA extraction kit	Institute of Physical Biology	SH-d		Mechanical cell disruption and DNA purification with detergent/phenol	3

Note: At hands-on time, the overnight pre-enrichment in BPW prior to extraction is depicted as ≥12h.
Legend: e, pre-enrichment; d, direct extraction; BPW, buffered peptone water

with PCR and calculated using the Most Probable Number Calculator program version 4.04 (http://www.epa.gov/microbes/mpn.exe) with four dilution and 20 tubes per dilution parameter settings.

Results

Differences in *Salmonella* detection limit could be observed between the two groups of DNA extraction kits based on the employment of sample pre-enrichment step. The overnight pre-enrichment prior to DNA extraction markedly improved the detection, since bacteria of the genus *Salmonella* were detected in all spiked water samples with some trivial discrepancies observed for higher bacterial loads with the method S-e (Table 2). Detection limit was at least 18 CFU/L, which was the lowest contamination level tested.

Table 2: Efficiencies of DNA extraction kits used in the present study for water samples inoculated with *Salmonella* Enteritidis (CFU/L)

		Number (%) of Salmonella-positive PCR reactions			
		pre-enrichment		direct extraction	
CFU/L	Number of tested PCR reactions	FP-e	S-e	AW-d	SH-d
18	20	20 (100)	20 (100)	0 (0)	0 (0)
36	20	20 (100)	20 (100)	2 (10)	4 (20)
72	20	20 (100)	19 (95)	4 (20)	11 (55)
144	20	20 (100)	19 (95)	12 (60)	20 (100)

Note: Samples were prepared in five parallels and for each parallel four PCR replicates were performed (i.e. 20 tested PCR reactions). See Table 1 for details on DNA extraction kit abbreviations

When comparing methods for DNA extraction not preceded with the additional manipulation after sample collection, differences in *Salmonella* detection limit were observed as well. Both methods for direct DNA extraction from water samples generated positive results at 36 CFU/L and/or at higher bacterial loads. However, all parallels and replicates tested positive only with SH-d for water samples spiked with 144 CFU/L (Table 2). In comparison to AW-d, SH-d supported a markedly higher number of positive results in all spiked samples. Likewise, when PCR results were quantified using the MPN method (Figure 1), the estimated level was significantly higher for the method SH-d in comparison to AW-d for the highest tested contamination level (144 CFU/L). Other contamination levels with the exception of the lowest one (18 CFU/L), where detection failed for both methods, showed no statistical difference ($P<0.05$). For the method SH-d, the estimated levels of contamination (MPN/L) were similar to the inoculated *Salmonella* loads (CFU/L).

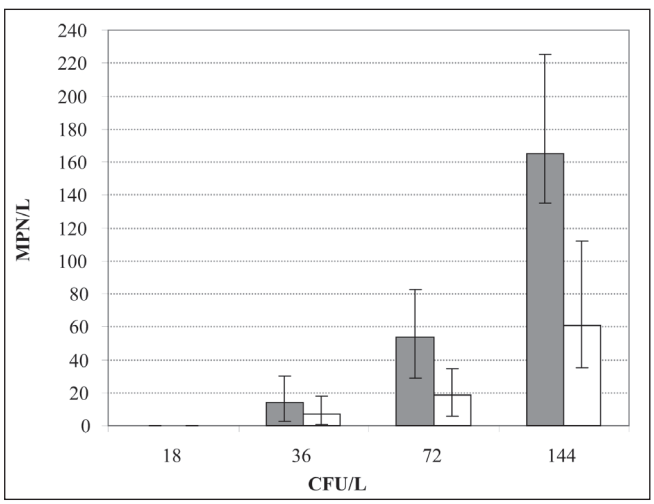

Figure 1: The inoculated *Salmonella* load (CFU/L) plotted against the estimated load (MPN/L) for water samples processed by the two direct DNA extraction methods: SmartHelix® Complex Samples DNA extraction kit (gray bars) and Adiapure® Water DNA extraction and purification kit (white bars); error bars represent 95% confidence levels

Discussion

Detection limit for *Salmonella* spp. in tap water samples employing the PCR amplification depended on the method for microbial DNA extraction. Bacterial enrichment in a selective or non-selective growth medium prior to molecular detection enables multiplication of targets to the detectable concentration, and it also dilutes the PCR inhibitory substances that can be present in the investigated samples. As expected, the overnight pre-enrichment step employed with the methods FP-e and S-e prior to molecular detection proved to be significant for lowering the detection limit of *Salmonella* spp. in comparison to direct methods. Increasing the number of samples in the future research will enable determination of the exact detection limit of direct extraction methods like SH-d, and the detection probability as reported before (16) will enable more objective calculation stating the expected relative frequency of positive PCR results at various contamination levels over a certain period of time.

Many PCR assays employed for spiked water, food, soil, and faecal samples included the pre-enrichment step that enabled detection of only few *Salmonella* cells per sample (17-19). However, when an instant response of the public health-assuring authorities is needed for the prevention of *Salmonella*-associated epidemic outbreaks, a protocol requiring the pre-enrichment step represents a major disadvantage. The methods for direct DNA extraction from environmental samples enable at least four to five time faster response, obtaining DNA within three hours after sampling, although not distinguishing between viable and dead bacterial cells. Samples can be processed immediately after collection and results obtained at the same day, rendering possible the prevention of dissemination of pathogens in water supplies.

The selected direct extraction methods differed to some extent in the required hands-on time, but more importantly in their efficiency. Results indicated that the method SH-d was more suitable than the other selected method for rapid detection of *Salmonella* spp. in tap water samples. It is suitable for DNA extraction from water samples for pathogen detection in the case of both high and low contamination levels. To optimize DNA extraction after sample filtration to concentrate microbial cells, the method SH-d was adapted to completely disrupt the filters and bacterial cell membranes using the mechanical force (*i.e.* beads were added to filtered samples prior to bead-beating disruption) in combination with detergent/phenol treatment to obtain high DNA extraction yields. As used in the method AW-d, chemical/thermal cell disruption without filter disintegration in combination with DNA purification columns proved to be less efficient for obtaining high DNA extraction yields possibly due to insufficient detachment of cells from

filters, therefore excluding a certain proportion of microbial cells from DNA extraction procedure. Our preliminary studies indicated that the mechanical disintegration increased the extraction yields from bacterial cells for at least five times, which was based on the measurement of DNA concentration (unpublished data). As strong mechanical forces can cause DNA fragmentation, subsequent PCR amplification can be less efficient. However, the duration of mechanical disruption was optimized to achieve complete disintegration of filters and cells but to obtain microbial DNA of satisfactory quality for PCR amplification.

The present study enabled the first insight into the applicability of the newly developed DNA extraction kit (SH-d). In addition to the present study, a preliminary research was conducted on spiked water samples collected from a pond, representing the naturally contaminated water source. For pond water, it could be observed that the sensitivity of detection was at least six times lower than for tap water when applying direct extraction methods but similar when including the pre-enrichment step (unpublished data). More research will be performed on raw water samples to study the effect of the background microbiota and the inhibitory chemical compounds. The collected data from our and other laboratories will enable us to introduce the potential method improvements. The practical operating range of PCR detection (16) preceded by the SH-d extraction will be determined and an internal amplification control (20) will be included if needed to give the newly developed kit a promising entry into the routine diagnostics. The analysis could be performed even faster without the DNA extraction step (21). However, the low contamination levels require a detection procedure of greater sensitivity and reproducibility. The extraction step employing a method not preceded with the pre-enrichment meets the demand for fast response.

We can conclude that direct methods for DNA extraction from bacterial cells concentrated on filters can be successfully used in place of procedures employing cultivation for the detection of *Salmonella* spp. in tap water samples. The new direct method SH-d, namely SmartHelix® Complex Samples DNA extraction kit developed in our laboratory, showed an efficient performance, therefore represents a good choice when rapid pathogen detection is needed for safe food and water assurance.

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PRIMERJAVA METOD OSAMITVE DNK ZA ODKRIVANJE BAKTERIJ IZ RODU *SALMONELLA* V PITNI VODI

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Povzetek: Bakterije iz rodu *Salmonella* predstavljajo splošen zdravstveni problem, povezan z okuženimi živili in fekalno onesnaženo vodo. Namen našega dela je bil s primerjavo različnih metod osamitve DNK ugotoviti učinkovitost in hitrost odkrivanja salmonel v vzorcih pitne vode, ki smo jim poprej dodali omenjene bakterije v različnih koncentracijah. Uporabili smo štiri različne metode osamitve DNK: komercialna kompleta foodproof™ Sample Preparation Kit I (Biotecon Diagnostics) in QIAamp® DNA Stool Mini Kit (Qiagen) za osamitev DNK po čeznočni obogatitvi ter kompleta Adiapure® Water DNA Extraction and Purification Kit (Adiagene) in SmartHelix® Complex Samples DNA Extraction Kit, ki smo ga nedavno razvili v našem laboratoriju, za direktno osamitev DNK. DNK smo uporabili za pomnoževanje v reakciji PCR s parom začetnih oligonukleotidov, ki je specifičen za rod *Salmonella*. Komplet SmartHelix se je za direktno odkrivanje DNK salmonel v vodi izkazal kot bolj učinkovit v primerjavi s kompletom Adiapure Water. Čeznočna obogatitev je izboljšala odkrivanje salmonel v vzorcih, ki smo jim dodali majhno koncentracijo bakterij, vendar pa je podaljšala čas analize. Rezultati kažejo na to, da bi morali na novo izdelani komplet SmartHelix upoštevati kot eno izmed možnih izbir, ko želimo v vzorcih vode patogene bakterije odkriti hitr,o z namenom preprečevanja izbruhov bolezni in zagotavljanja varne hrane.

Ključne besede: osamitev DNK; varna hrana; odkrivanje patogenov; PCR; *Salmonella*; voda

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Original Scientific Article

INVESTIGATION OF TWO *SALMONELLA* SEROVAR ENTERITIDIS OUTBREAKS USING THE PULSED-FIELD GEL ELECTROPHORESIS: A GOOD EXAMPLE OF COLLABORATION AT THE NATIONAL LEVEL

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Summary: *Salmonella* is an important zoonotic pathogen in animals and humans. In the European Union, *Salmonella enterica* subspecies *enterica* serovar Enteritidis (serovar Enteritidis) is one of the serovars most frequently associated with human illness. The most important food vehicles responsible for the infection are eggs and egg products. We describe two serovar Enteritidis outbreaks on account of consumption of contaminated eggs. The first outbreak due to vanilla cream served as dessert in a restaurant involved 36 persons. As the eggs used in preparing the vanilla cream were no longer available for examination, an indirect epidemiological link between the infected laying hen flock and humans was demonstrated by testing the faeces and dust samples from the relevant laying hen flock. In the second outbreak, two persons developed a severe form of salmonellosis after having consumed fried eggs. A sample of eggs taken from the same laying hen flock as the eggs consumed by the two patients tested positive for serovar Enteritidis.

Isolates from both the outbreaks were subjected to molecular typing for the assessment of genetic relatedness. Pulsed-field gel electrophoresis (PFGE) revealed that the profiles of the majority of isolates from the same outbreak were indistinguishable and should therefore be considered to represent the same strain.

This is the first molecular epidemiological investigation of serovar Enteritidis outbreaks in Slovenia that involved the public health and veterinary authorities and as such set a good example of collaboration of different national services.

Key words: *Salmonella* serovar Enteritidis; salmonellosis; outbreak; genotyping; laying hens; eggs; humans

Introduction

Salmonella has long been recognised as an important zoonotic pathogen of economic significance in animals and humans. A common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals, which results in a variety of food of animal and plant origin as sources of infections. Transmission often occurs when organisms are introduced in food preparation areas and allowed to multiply in food, either due to inadequate storage temperatures, inadequate cooking or cross-contamination of ready-to-eat food (1).

In humans, the symptoms of salmonellosis are often mild and most infections are self-limiting, lasting a few days. However, in patients with primary and secondary disorders of the immune response and in patients with severe underlying diseases, the infection may be more serious. In animals, sub-clin-

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ical infections are common. *Salmonella* may easily spread undetected between animals which may become intermittent or persistent carriers (1).

In 2009, *Salmonella* was the predominant causative agent of reported food-borne outbreaks in the European Union (EU). The most important food vehicles in the outbreaks with known causative agent were eggs and egg products, mostly associated with *Salmonella enterica* subsp. *enterica* serovar Enteritidis (serovar Enteritidis) contamination. Serovar Enteritidis was the most frequently reported serovar (52.3% of all known serovars in human cases) (1).

For efficient preventive and control measures, it is of utmost importance to determine the sources of infection and routes of transmission. Pulsed-field gel electrophoresis (PFGE) has been shown to be appropriate for epidemiological studies of *Salmonella* (2-6). It has been used as the principal method for subtyping salmonellas in the USA since 1996 (7) and successfully applied to outbreak investigations due to serovar Enteritidis (8-10). The method enables the assessment of genetic relatedness among the isolates and, together with supporting epidemiological data, provides the possibility to detect links between isolates from different sources.

The objective of this paper is to report on the investigation of two serovar Enteritidis-related outbreaks recorded in 2009 and to demonstrate the importance of good collaboration between the public health and veterinary professionals.

Materials and methods

Investigation of Outbreak No 1

In March 2009, an anonymous report of food-borne intoxication was received by the Public Health Inspectorate of the Republic of Slovenia. At the same time, the Clinical Microbiology Laboratory of the Institute of Public Health of Novo mesto (IPH–NM) reported cases of serovar Enteritidis isolation to the epidemiological services. This was the basis for conducting an epidemiological investigation in order to determine the source of infection. Inquiries revealed that intoxication had presumably occurred on 15 March 2009 in a restaurant in the Dolenjska region. Relevant meal was consumed by 30 restaurant customers, six restaurant employees and their family members. Sanitary examination of the kitchen took place and the staff was instructed to follow the guidelines for prevention of transmission of enteric contagious disease and to carry out the cleaning and disinfection measures. Surface swabs were taken and microbiological examination of stool samples of the diseased customers, kitchen staff and family members was conducted.

Two stool samples of patients were examined at the beginning of the outbreak and both tested positive for serovar Enteritidis. After the reported outbreak, 17 stool samples of persons connected with the outbreak (restaurant employees, customers and contact persons) were examined. Serovar Enteritidis was detected in three persons, including two restaurant employees. Only one patient was hospitalised, and there were no fatalities. Surface swabs were all negative for serovar Enteritidis. Five serovar Enteritidis isolates were sent to the Institute of Public Health of Celje (IPH–CE) for PFGE typing. PFGE profiles of all the isolates were identical.

Interviews with patients showed that vanilla cream (made of fresh eggs and used as topping on canned fruit) was the possible source of infection. The dessert was consumed by all the persons that later developed the enteric disease. Vanilla cream was not subjected to testing as it was no longer available. However, the Public Health Inspectorate managed to detect the origin of the eggs and notified the competent Regional Office (RO) of the Veterinary Administration of the Republic of Slovenia (VARS) of a suspected outbreak of salmonellosis caused by food of animal origin, indicating all the particulars of the relevant food business operator (FBO). The competent VARS RO conducted official control in the establishment of the FBO involved in egg production, taking two faeces samples and a single dust sample in the relevant laying hen flock, as required by the applicable regulations.

The National Veterinary Institute (NVI) detected serovar Enteritidis in the dust and faeces samples. The competent VARS RO instituted the required measures in the relevant FBO's establishment, including the ban on placing table eggs on the market and on any movements of the animals, except for killing or slaughter.

Investigation of Outbreak No 2

In the first five months of 2009, an unusually high number of persons (n=51) suffering from enteric disease caused by serovar Enteritidis were detected by the Institute of Public Health of Kranj (IPH–KR), as compared to a 10-year average of 15 cases within a period between January and May. Three family outbreaks of salmonellosis were investigated in the first

half of 2009. Epidemiological investigation revealed that infections were predominantly linked to consumption of raw or insufficiently heat-treated eggs or inadequately prepared egg dishes in the patients' home environment. Due to delayed notification of the two family outbreaks, microbiological examination of food samples was no longer possible and, due to a small number of affected persons, analytical epidemiological studies could not be performed.

This case describes a family outbreak due to consumption of fried eggs, which caused a severe form of salmonellosis in a 13-month-old child and his 36-year-old mother in May 2009. Both the patients were admitted to hospital. Serovar Enteritidis was detected in their stool samples. The isolates were PFGE typed at IPH–CE, and shared a common PFGE profile.

IPH–KR notified VARS of a salmonellosis outbreak and of a suspected source of infection. A single egg originating from the same batch as table eggs involved in the outbreak was brought to VARS RO by a member of the affected family. VARS was able to trace back the laying hen house by the producer's identification imprinted on the egg. The competent VARS RO conducted official control in the establishment of the FBO involved in egg production, taking two faeces samples and a single dust sample in the relevant laying hen flock, as required by the applicable regulations, and submitting a sample of eggs for *Salmonella* testing to the NVI. Serovar Enteritidis was identified in all the samples. The competent VARS RO instituted the required measures in the FBO's establishment as referred to above. As a precautionary measure, the media were requested by IPH–KR to disseminate information among the general public on the safety of use, preparation and consumption of raw eggs and egg dishes.

In June 2009, NVI received a request from VARS to compare the PFGE profiles of isolates involved in the two outbreaks. PFGE profiles of human isolates were obtained from IPH–CE and compared with the PFGE profiles of non-human isolates in the NVI database.

Microbiological examination

At suspected epidemic of an enteric contagious disease, samples are investigated for the presence of most common bacterial, viral and parasitic agents.

Accordingly, stool samples were inoculated both into enrichment medium for enterobacteria (selenite broth) and onto selective media for other intestinal pathogens (Karmali agar, sMaC agar, Drigalski agar, blood agar). After 24 hours, they were transferred from enrichment onto *Salmonella* selective medium (XLD), which was checked for suspect colonies. These were subsequently identified to the species/serotype level by biochemical tests and agglutination.

Dust and animal faeces were processed according to EN/ISO 6579-2002/Amd1:2007. After 18 ± 2 hours' enrichment in buffered peptone water, they were inoculated onto modified semisolid Rappaport-Vassiliadis agar and, in case of swarming, the culture was inoculated onto XLD and Rambach agar. *Salmonella*–suspect colonies were identified by biochemical tests and agglutination. The sample of eggs was tested according to ISO 6579:2002.

PFGE typing

Human isolates were sub-typed by PFGE at IPH–CE that performs the nation-wide PFGE typing of isolates from human outbreaks. Isolates from dust, eggs and hen faeces were sub-typed at the NVI. Details on isolates subjected to analysis of genetic relatedness are given in Table 1. A standardised protocol was used for PFGE typing, using the restriction endonuclease *Xba*I (11). Fragments generated were separated by electrophoresis for 20 hours at 6 V/cm and 14 °C, and with pulse times from 2 s to 64 s in a CHEF-DR II system (BioRad, Hercules, CA, USA). PFGE profiles were subjected to computer-assisted analysis with BioNumerics software (version 5.0, Applied Maths, Sint-Martens-Latem, Belgium). Serovar Braenderup strain H9812 was used for normalisation. Dendrograms were created using an UPGMA (Dice coefficient) algorithm.

Table 1: Origin of serovar Enteritidis isolates involved in PFGE typing

Event	Origin and number of isolates			
	Man	Laying hen	Dust	Egg
Outbreak 1	5	1	1	0
Outbreak 2	2	2	1	1[a]

[a] pooled sample of 10 eggs

Results

PFGE typing

Dendrogram generated from seven isolates related to the first outbreak revealed identical PFGE pro-

files for six isolates, whilst the profile of one isolate from dust differed in one band.

Similar results were seen when comparing genotyping results of isolates involved in the second outbreak. All isolates shared a common profile, with the exception of the one obtained from a laying hen that exhibited a one-band difference (Figure 1).

Discussion

Different *Salmonella* serovars have been related specifically with some foods. Serovar Enteritidis is particularly related to eggs (12, 13). This is linked to the ability of this serovar to persistently colonise the avian reproductive tract, resulting in internally contaminated eggs, and in egg shell contamination (14). According to the European Food Safety Authority (EFSA) report for 2009, *Salmonella* was only found in a very a low proportion of table eggs and egg products, at levels of 0.5 % and 0.6 %, respectively. However, *Salmonella* was still the most frequently reported cause of food-borne outbreaks and the main food vehicles were eggs and egg products (1).

A key factor enabling the egg to be an efficient vehicle for human infection is the manner in which people handle and eat eggs. Eggs are one of the few animal products that are frequently eaten raw or undercooked. Hedberg et al. (15) found that patients with sporadic serovar Enteritidis were over five times more likely to have eaten raw or undercooked eggs in the three days before their illness, compared with healthy control subjects. The extent to which eggs were not cooked was directly associated with illness. In the second outbreak described herein,

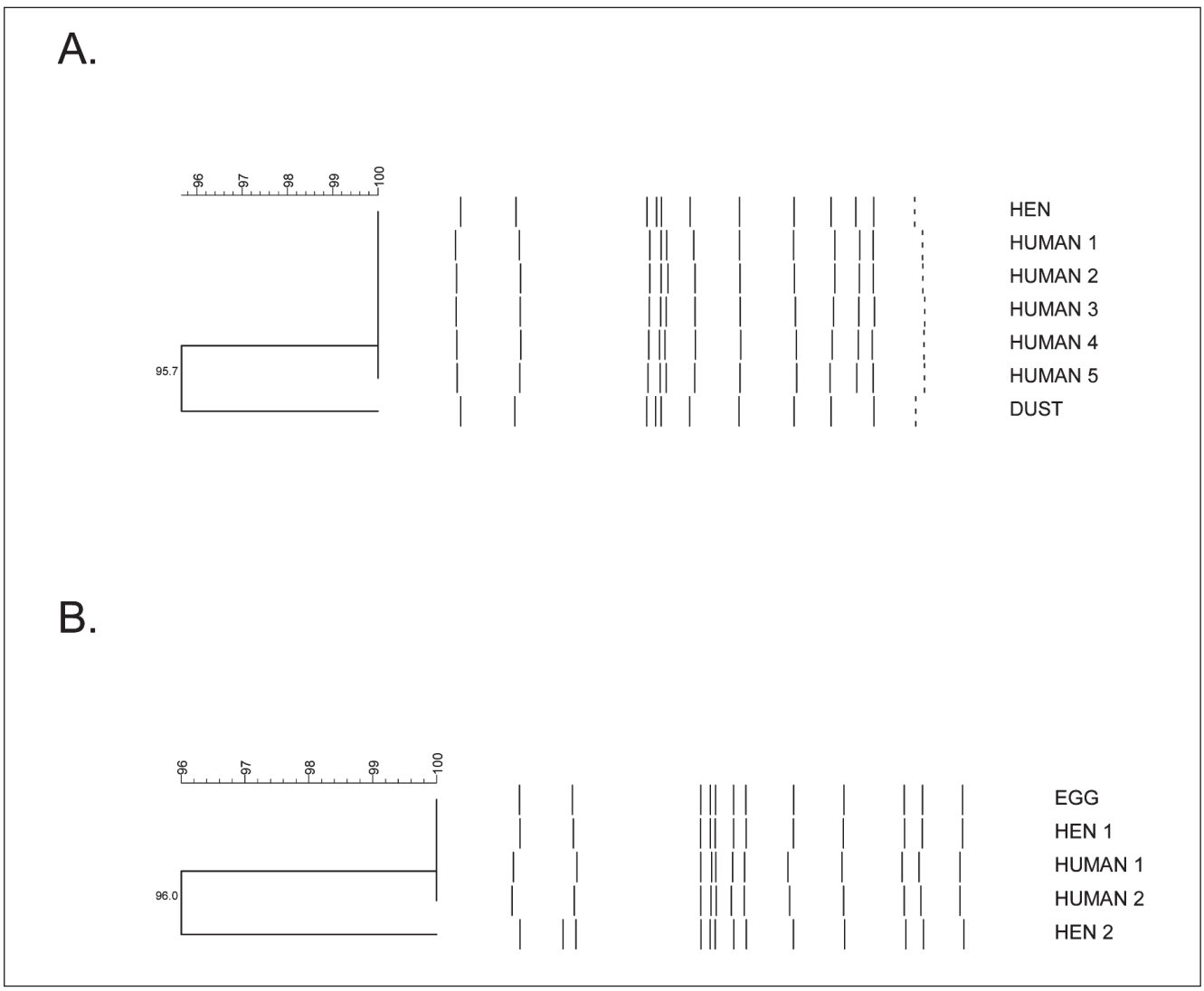

Figure 1: Dendrograms showing genetic relatedness of serovar Enteritidis isolates related to the first (A) and the second (B) outbreak

two persons suffered from severe form of salmonellosis due to consumption of fried eggs. It has been reported before that fried eggs may be a source of infection with serovar Enteritidis (16), especially if when eaten, the egg white and yolk are still soft or runny. Infection with serovar Enteritidis is also associated with eating outside the home (17). One reason that commercial or other large-scale food preparation settings may be more frequently associated with illness is the practice of pooling large numbers of eggs for use in egg dishes (18). Thus, one egg can contaminate a large amount of food and endanger a large number of consumers. The first outbreak described in this paper was an example of infection acquired in a restaurant. Eight out of 36 exposed persons developed symptoms of enteric disease and serovar Enteritidis was isolated from five patients.

The numbers of human salmonellosis cases reported in the EU continued to decline in 2009 as a part of a statistically significant trend since 2005. The reduction was particularly substantial for the most frequently reported serovar Enteritidis. It is assumed that the observed reduction of salmonellosis cases is mainly due to successful *Salmonella* control programmes in fowl populations (1). However, the risk of infection with serovar Enteritidis still persists and should not be neglected or underestimated due to potential complications of the disease. Early epidemiological and microbiological investigation is of utmost importance for preventing the transmission of infection and molecular typing is essential for detection of *Salmonella* source.

PFGE method has been proven to be useful for assessing the relatedness of serovar Enteritidis isolates and for investigating the outbreaks as a gold standard (2, 19). PFGE has been used for typing animal and food isolates of *Salmonella* at the NVI for several years. Previously established collaboration with IPH–CE and the use of standardised PFGE protocol undoubtedly contributed to fast and efficient work on comparison of PFGE profiles. Comparison revealed that profiles of the majority of isolates from the same outbreak were indistinguishable and should therefore be considered as a single strain. In both the outbreaks, single isolates showed a one-band difference compared to the other isolates. These isolates should be considered as closely related to the outbreak strain by the criteria for bacterial strain typing proposed by Tenover et al. (20).

Even though PFGE has been widely used for characterisation of genetic relatedness of serovar Enteritidis isolates and investigation of outbreaks, it has been suggested to have limited value in epidemiological analysis because of the high genetic homogeneity among strains of serovar Enteritidis (21, 22). On the other hand, some comparative studies indicate that isolates that are indistinguishable by PFGE are unlikely to demonstrate substantial differences by other typing techniques (e.g. 23). Nevertheless, it has been suggested that a single method cannot be used reliably for epidemiological analysis of unrelated and related strains of serovar Enteritidis (24-26). This vantage point should be borne in mind at future introduction of additional genotyping methods.

This is the first molecular epidemiological investigation of serovar Enteritidis outbreaks in Slovenia that involved public health and veterinary professionals, setting a good example of collaboration between different national authorities. Coordinated action resulted in successful investigation of the outbreaks. Constant high-level cooperation and introduction of new typing methods with increased discriminatory power constitute a good basis for the effective prevention and control of zoonoses in general. In the future, typing of all serovar Enteritidis isolates obtained from routine laboratory examinations would be reasonable as it would allow us to identify genotypes with a high spreading potential, to trace the source of epidemic strains, to detect large epidemics, and to follow the rapidity of spread of certain genotypes within the food chain.

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UPORABA PULZNE ELEKTROFOREZE V PREISKAVI DVEH IZBRUHOV, POVZROČENIH S SALMONELO SEROVARA ENTERITIDIS: DOBER PRIMER SODELOVANJA NA DRŽAVNI RAVNI

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Povzetek: Salmonela je pomembna patogena bakterija pri živalih in ljudeh. *Salmonella enterica* subsp. *enterica* serovar Enteritidis (serovar Enteritidis) spada med serovare, ki v Evropski uniji najpogosteje povzročajo obolenja ljudi. Najpomembnejši vir okužbe med živili predstavljajo jajca in jajčni izdelki. V prispevku opisujemo dva izbruha, povzročena s salmonelo serovara Enteritidis, zaradi zaužitja kontaminiranih jajc. Pri prvem izbruhu se je 36 ljudi okužilo z vanilijevo kremo, postreženo kot sladico v restavraciji. Ker jajca, iz katerih je bila krema pripravljena, niso bila več na voljo, je bila s preiskavo iztrebkov in prahu iz reje kokoši nesnic ugotovljena zgolj posredna epidemiološka povezava med rejo kokoši nesnic in ljudmi. Pri drugem izbruhu sta dve osebi zboleli za hujšo obliko salmoneloze, ki je bila posledica zaužitja ocvrtih jajc. Vzorec jajc iz iste reje kokoši, iz katere so izvirala zaužita jajca, je bil pozitiven na serovar Enteritidis. Z molekularno tipizacijo smo ugotavljali gensko sorodnost izolatov iz obeh izbruhov. Rezultati pulzne elektroforeze so razkrili, da so bili profili večine izolatov iz posameznega izbruha enaki in so torej predstavljali isti sev.

V Sloveniji je to prva molekularno-epidemiološka preiskava izbruhov, povzročenih s serovarom Enteritidis, pri kateri so sodelovali strokovnjaki javnega zdravstva in veterinarske medicine, in predstavlja dober primer sodelovanja med različnimi državnimi službami.

Ključne besede: *Salmonella* serovar Enteritidis; salmoneloza; izbruh; genotipizacija; kokoši nesnice; jajca; ljudje

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Case Report

INCLUSION BODY HEPATITIS ASSOCIATED WITH FOWL ADENOVIRUS TYPE 8b IN BROILER FLOCK IN SLOVENIA – A CASE REPORT

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Summary: Inclusion body hepatitis (IBH) is an acute disease in chickens caused by fowl adenoviruses (FAdVs). Among twelve known FAdV types (FAdV-1 to 8a, and FAdV-8b to 11), classified into five different species (A-E), all of them were already involved in naturally occurring cases. The disease is mainly distributed in areas with intensive poultry industry. In Slovenia the etiological agent of IBH has not been confirmed to date.

An outbreak of acute mortality affected a broiler flock of 12,000 animals. In two waves of elevated mortality rate, a total of 264 (2.2%) chickens were found dead in one week time. Affected birds showed ruffled feathers, depression, watery droppings and some of them limping. The most common pathological lesions seen on necropsy were pale, swollen and friable livers with subcapsular hemorrhages. On histological examination, acute hepatitis characterized by necrosis and hepatocytes, with large basophilic intranuclear inclusion bodies, were observed. The histological results were characteristic for IBH caused by adenovirus infection. The causative agent was identified as fowl adenovirus (FAdV) type 8b, a member of the Fowl adenovirus E species, based on PCR results of partial sequence of adenoviral polymerase and hexon gene. The confirmed type in our case is one of the most common causative agents involved in IBH. In addition, infectious bursal disease virus and infectious bronchitis virus were detected in the same flock.

Key words: inclusion body hepatitis outbreak; fowl adenovirus 8b; broilers; Slovenia

Introduction

Fowl adenoviruses (FAdVs) are a very heterogeneous group of viruses. Twelve types (formerly serotypes), named FAdV-1 to 8a, and FAdV-8b to 11, are classified into five different species (A-E) (1). They are believed to be ubiquitous in poultry farms (2). Not all FAdVs are considered to be pathogenic for chickens but every type has already been recovered from naturally occurring cases of inclusion body hepatitis (IBH) (3, 4).

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IBH was first described in the USA in 1963 and then rapidly spread over the world (5). Its importance in the poultry industry has been increasing in recent years (2). A sudden onset of increased mortality may reach 10% in 3-4 days and usually returns to normal after 5 days from the onset of clinical signs. If there are secondary bacterial infections ongoing contemporarily, mortality can reach 30% and can continue for several weeks (5, 6, 7, 8, 9). The severity of the disease may also depend on some other predisposing factors that enhance the pathogenic potential of FAdV infection, such as a poor environment and management (9, 10). It has been proven that the initial involvement of immunosuppressive agents, including infectious bursal disease virus

(IBDV) and chicken anemia virus (CAV) or some mycotoxins, such as aflatoxins, is needed for IBH onset (10, 11, 12, 13, 14).

On the other hand, different FAdV types, called highly pathogenic FAdVs, have been considered to be the primary pathogen in IBH. Among them, FAdV-8 and 9 are the most commonly detected ones (3, 9, 15, 16, 17) and seem to predominate in some areas (3, 17).

The present paper is the first report of IBH with detailed clinical and laboratory findings in an outbreak in commercial broilers in Slovenia.

Material and methods

Case history

Acute mortality was observed in a broiler flock of 12,000 animals. The birds were housed as day-old chickens and were vaccinated against Newcastle disease (ND) on day 6 (Pestikal La Sota SPF, Veterina, CRO), IBD on day 13 (Nobilis Gumboro 228E, Intervet/Schering-Plough, USA) and infectious bronchitis on day 16 (Nobilis IB 4-91, Intervet/Schering-Plough, USA). At the age of 17 days, 146 chickens (1.22%) were found dead at random throughout the house. The flock was treated with amoxicillin (Paracilin, Intervet/Schering-Plough, USA) for 5 days. In the six days following the first onset, 118 chickens (0.98%) died. Affected birds showed ruffled feathers, depression, watery droppings and limping.

Fourteen broiler chickens that died at the age of 23 days were submitted to the Institute of Poultry Health, Veterinary Faculty (VF), University of Ljubljana on the day of death.

The overall production results in the affected flock were comparable to those obtained in other broiler flocks. The mortality was 2.90%. Nonetheless, a higher feed conversion rate (1.94 kg compared to a predicted 1.88 kg) was obtained and the average body weight at the age of 34 days was higher than expected; 1.70 kg compared to 1.50 kg planned.

Gross and histopathological examinations

Pathologic examinations were performed on all birds submitted. Tissue blocks of the liver, kidney, heart and spleen were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 µm and stained with hematoxylin and eosin for light microscopy. Histopathology was performed at the Institute of Pathology, Forensic and Administrative Veterinary Pathology, VF.

Virological examinations

For molecular investigations, DNA and RNA were extracted from material taken at necropsy and frozen until investigation. Portions of liver and spleen were separately homogenized in phosphate buffered saline (PBS), prepared as a 10–20% w/v suspension. DNA was extracted by using a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA was extracted from trachea and cloacal swab by using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Before RNA extraction, 2 ml of PBS was added to each swab and vortexed vigorously.

Detection of FAdV

Attempts to detect adenoviruses in liver and spleen tissue were made by using nested polymerase chain reaction (PCR) with degenerate, consensus primers targeting the viral DNA dependent DNA polymerase gene, as described by Wellehan et al. (18). In addition, more specific, degenerate primers hexon A and hexon B that amplify a region of the hexon gene were used for establishing FAdV serotype, as reported previously (19).

Detection of CAV

For detection of CAV in liver and spleen tissue, primers that target the highly conserved gene encoding VP2 were used, as described by Noteborn et al. (20).

Detection of IBDV

For detection of IBDV from cloacal swab, a reverse transcription (RT)-PCR method was applied, as described previously (21), using primers specific to the genome fragment that codes for the hypervariable region of VP2 (22).

Detection of infectious bronchitis virus (IBV)

The presence of the IBV genome in tracheal and cloacal swabs was tested by RT-PCR. Primer pair CK2/CK4 targeting the variable region of S1 gene was used, as described by Keeler et al. (23).

PCR product analysis and typing

PCR products were visualized by electrophoresis on a 1.8% ethidium bromide stained agarose gel. The PCR products were excised and purified with Wizard PCR Preps DNA Purification System

(Promega, Madison, Wi, USA) and sent for sequencing purposes to Macrogen DNA Sequencing Service (South Korea). Sequence analyses were performed by DNAStar (DNAStar Inc., USA) and NCBI BLAST Tools (http://www.ncbi.nl.nih.gov). Multiple protein alignments were made using the ClustalW program. The alignments were reverted back to the saved DNA-sequence and edited using BioEdit. Phylogenetic calculations were performed using the Phylip package online (Mobyle@pasteur: http://mobyle.pasteur.fr) by Dnadist with the Kimura two parameters model. The Fitch program was used by the Fitch–Margoliash method with global rearrangements for phylogenetic tree reconstruction. The trees were visualized using Mega.

Isolation of FAdV

For adenovirus isolation, 8-day-old SPF embryonated chicken eggs (Lohman, Cuxhaven, Germany) were used. Liver homogenate was prepared and inoculated into the egg yolk, as described previously (24). Briefly, liver tissue was homogenized. Ten percent liver solution was made adding the minimum essential medium and penicillin streptomycin solution and centrifuged at 1500×g for 10 minutes. A total of 0.1 ml of the supernatant was used for each inoculation. Inoculated eggs were observed by candling daily. All dead embryos were necropsied. Livers were taken for histopathology and molecular examinations for FAdV detection, as described above.

Bacteriological examinations

Routine bacteriology was performed on liver samples. Samples were cultured aerobically at 37°C on 5% sheep's blood and Drigalski agar plates. Cultures were considered negative if no growth was detected after a 48-hour incubation period.

Serological examinations

For serological investigation, 20 blood samples were taken on the 36th day of age. Antibodies against IBV, CAV and IBDV were tested by enzyme-linked immunosorbent assays (ELISAs) (IDEXX, Westbrook, ME), according to the manufacturer's instructions.

Results and disscusion

Necropsy and histopathology results revealed pathological changes characteristic for IBH, as described previously (2, 5, 6). Predominating gross lesions seen were pale, swollen and friable livers, kidneys with subcapsular petechial hemorrhages and pale myocardium. Occasionally, mild tracheitis and catarrhal enteritis were noticed. All examined birds were in good body condition. Microscopic examination revealed acute hepatitis, with randomly distributed multifocal areas of acute necrosis as well as numerous disseminated hepatocytes with large basophilic intranuclear inclusion bodies scattered among necrotic hepatocytes (Figure2). Multiple subcapsular hemorrhages, multifocal groups of hepatocytes with lipid degeneration, and cholestasis were also present. Similar large intranuclear basophilic inclusion bodies as in the liver and karyorrhexis were found in the red pulp cells of the spleen and in the tubular cells of the kidneys but were less frequent than in the liver (Figure3).

Livers and spleens gave a positive PCR result for FAdVs. The PCR targeting the adenoviral DNA dependent DNA polymerase gene resulted in 321-bp-long products, the PCR targeting the hexon gene in 817-bp-long products. The determined partial hexon gene sequences from liver and spleen samples were found to be 100% identical on the nucleotide level (results not shown). According to the phylogenetic tree, the newly detected virus could be classified as FAdV type 8b, a member of the Fowl adenovirus E species (Figure1). Based on literature data, FAdV-E type 8b is one of the most common causative agents involved in IBH (9, 15, 17). The nucleotide sequences described in the present paper were submitted to GenBank and assigned accession numbers JF766220 for DNA polymerase gene and JF766221 for hexon gene, respectively.

Officially accepted adenovirus species names are given in italics. The topology of the tree was tested by bootstrapping. Bootstrap values are given for 1000 datasets if over 750. 1407: the adenovirus strain studied in this paper; FaAdV-1: falcon adenovirus 1; FAdV-1–11: fowl adenovirus 1–11; TAdV-1: turkey adenovirus 1. Accession numbers in the NCBI GenBank and strain names if applicable: 1407: JF766221; FaAdV-1: AY683541; FAdV-1: AC 000014, CELO; FAdV-2: AF508946, SR48; FAdV-3: AF508949, 75; FAdV-4: AF508950, 506; FAdV-5: AF508953, 340; FAdV-6: AF508954.2, CR119; FAdV-7: AF508955, YR36; FAdV-8a: AF508957, 58; FAdV-8b: AF508958.2, 764; FAdV-9: AC 000013, A-2A; FAdV-10: U26221; FAdV-11: EU979378, UF71; TAdV-1: GU936707, D90/2.

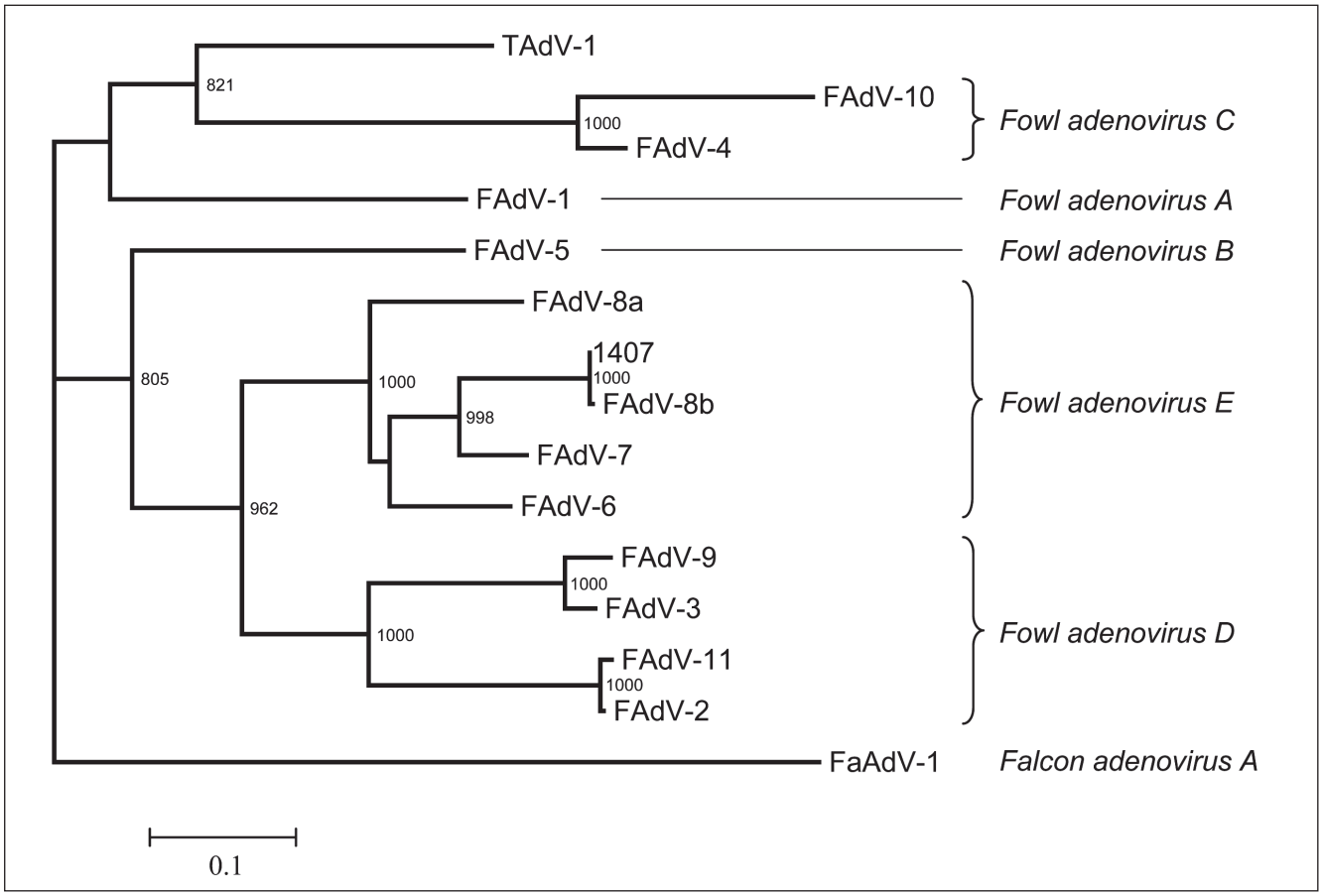

Figure1: Phylogenetic tree showing the distance matrix analysis of partial hexon gene DNA sequences from aviadenoviruses

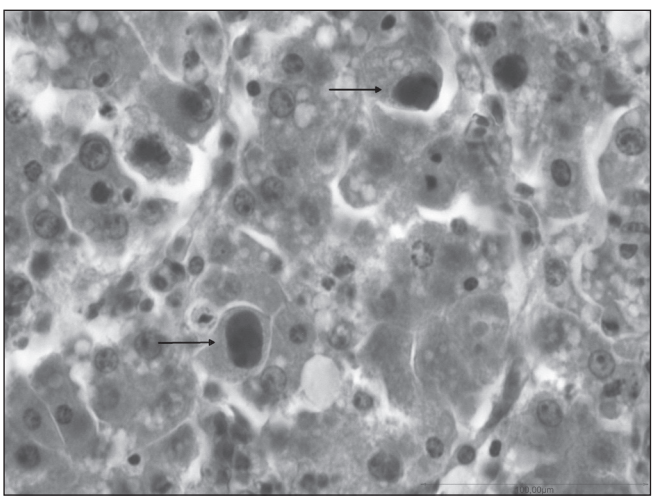

Figure2: Hepatocytes with large basophilic intranuclear inclusion bodies (arrows) (400×)

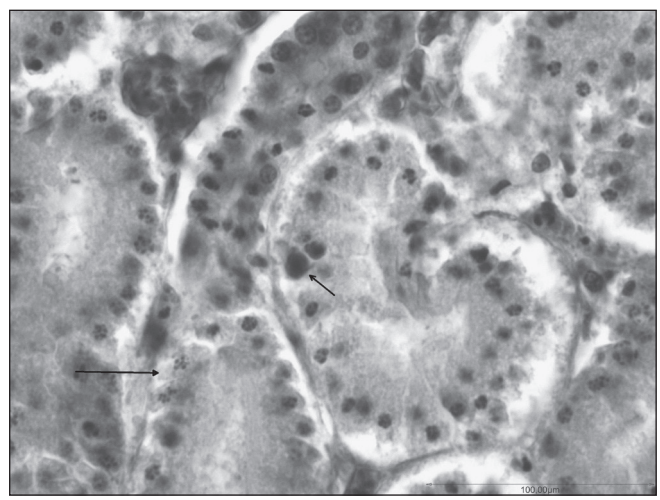

Figure3: Large intranuclear basophilic inclusion body (short arrow) and karyorrhexis (long arrow) in the tubular cells of the kidney (400x)

The infection was also confirmed by virus isolation. All embryos inoculated with liver homogenate of affected chickens died by day 5 post inoculation. On histological examination, acute hepatitis with distortion of the liver plates, multiple necrotic areas and disseminated individual necrotic hepatocytes was diagnosed. Variation of the nuclear size was evident in the hepatocytes of affected embryos but no inclusion bodies were found (results not shown). The presence of FAdV-8b was detected by PCR from the liver tissue of the embryos.

The severity of AdV-caused IBH can vary in broiler chickens. In general, it is believed that for the development of the clinical disease, most FAdVs need some immunosuppressive agents, which may trigger the mechanism. CAV and IBDV have most often been found to be predisposing factors to IBH outbreaks (10, 13, 14). On the other hand, many cases of IBH with highly pathogenic AdV types as primary pathogens have also been described (3, 9, 15, 17).

In our case, in addition to FAdV-8b, the presence of IBDV but not CAV was detected. The RT-PCR performed to test IBDV in cloacal swabs resulted in a product of appropriate size of approximately 630-bp. However, direct sequencing of the PCR product from the VP2 gene failed to give unambiguous results. A concurrent infection with vaccine and field strains of IBDV apparently occurred, resulting in heterogeneous PCR products that could not be sequenced without prior molecular cloning. Serological testing at the age of 36 days revealed an antibody response to IBDV (Table 1) originating from vaccination or/and from field infection. Since Zorman Rojs et al. (25) obtained significantly lower antibody titers (from 102 to 518) detected by the same (IDEXX) ELISA system in non-infected broilers vaccinated with intermediate plus vaccine, field infection is most likely in the present case. Infection with pathogenic strains of IBDV, including some less attenuated vaccine strains, has well-known immunosuppressive effects in chickens and might induce the development of IBH (10, 11, 12).

In addition, the presence of IBV in the trachea was confirmed. Its occurrence in broiler, broiler breeder and layer flocks in Slovenia has been studied extensively lately by Krapež et al. (26). Further molecular investigation showed that strain QX was involved (accession number GU 564331). Tracheitis, found at necropsy in some of the submitted chickens, might have been caused by the IBV infection. Presumably it did not have a significant influence on the course of IBH in our case. Interestingly, Ojkić et al. (9) found that co-infections with other viruses (IBDV, IBV and

Table 1: Summary of virological and serological results in IBH affected broiler flock

Virus	Virological examinations			Serology
	Molecular method (targeted region)	Sample	Result	IDEXX ELISA titer ± SD (Number of ELISA positive birds/ Number of tested birds)
FAdV	Nested PCR (polymerase gene)1	Liver	+	Not done
		Spleen	+	
	PCR (hexon gene)2	Liver	+	
		Spleen	+	
IBDV	RT-PCR (VP-2 region)3	Cloacal swab	+	3783 ± 893 (19/19)
IBV	RT-PCR (S-1 gene)4	Tracheal swab	+	1326 ± 650 (18/19)
		Cloacal swab	-	
CAV	PCR (VP-2 gene)5	Liver	-	1.148 ± 0.103 (0/19)*
		Spleen	-	

[1] Primers designed by Wellehan et al, 2004
[2] Primers designed by Meulemans et al, 2001
[3] Primers designed by Cao et, 1998
[4] Primers designed by Keeler et al, 1998
[5] Primers designed by Noteborn et al, 1992
* Blocking ELISA was used

reovirus) were more frequent in FAdV infections not related to IBH.

Some FAdV types have been described as inducing immunosuppression in chickens without any other predisposing factors. For instance, Schonewille et al. (27) demonstrated by experimental infection that FAdV-4 caused a depletion of B- and T-lymphocytes in lymphoid organs in SPF chickens. Immunosuppressive effect of confirmed FAdV type-8b was not evaluated. Moreover, it is not clear if the presence of IBDV detected in our case was the trigger mechanism for IBH outbreak or isolated AdV type-8b could produce the disease by itself.

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IZBRUH INKLUZIJSKEGA HEPATITISA PRI BROJLERJIH POVZROČEN Z ADENOVIRUSOM (FAdV) TIPA 8b

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Povzetek: Inkluzijski hepatitis je akutna bolezen piščancev, ki jo povzročajo kokošji adenovirusi (angl. fowl adenovirus, FAdV). Poznanih je 12 tipov adenovirusov (FAdV-1 do 8a in FAdV-8b do 11), ki so razvrščeni v 5 vrst (A-E). Vsi navedeni tipi so že bili dokazani v naravno potekajočih primerih inkluzijskega hepatitisa. Bolezen se pojavlja na področjih z razvito intenzivno rejo perutnine, v Sloveniji pa povzročitelj še ni bil dokazan.

V jati 12.000 piščancev brojlerjev je v razmiku tedna dni prišlo do nenadnega povečanja pogina. Poginilo je 264 (2,2 %) živali. Prizadete živali so bile depresivne, apatične, imele so drisko, nekatere izmed njih so šepale. Pri raztelesbi smo ugotovili izrazite spremembe na jetrih, ki so bila blede barve, povečana, krhke konsistence, z vidnimi subkapsularnimi krvavitvami. Histološke preiskave so potrdile akutni hepatitis, z nekrozami in znotrajceličnimi bazofilnimi inkluzijskimi telesi v hepatocitih. Histološki rezultati so bili značilni za adenovirusni inkluzijski hepatitis. Z molekularno metodo verižne reakcije s polimerazo (PCR), ki pomnožuje odsek adenovirusne polimeraze in odsek gena za hekson adenovirusov, smo določili povzročitelja. Dokazani etiološki agens je bil FAdV vrste E, tip 8b, ki je tudi eden izmed najpogosteje opisanih povzročiteljev inkluzijskega hepatitisa. Poleg omenjenega virusa smo v oboleli jati dokazali tudi sočasno prisotnost gumborske bolezni in kužnega bronhitisa.

Ključne besede: izbruh IBH; FAdV8b; brojlerji; Slovenija

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Namen ustanovitve in delovanja podjetja MD svetovanje d.o.o. je pomagati podjetjem pri poslovanju z nudenjem produktov in storitev, ki ne spadajo v osnovno dejavnost podjetja. To dosegamo s celovito ponudbo predstavljenih produktov in storitev.

Zato smo naš moto Skupaj bomo uspešnejši! nadgradili še z motom in sloganom Vse za Vas na enem mestu!

Vizija

Postati vodilna neodvisna družba s celotno ponudbo za podjetja in posameznike na enem mestu in na ta način prihraniti podjetjem in posameznikom čas in denar.

Vse to nam bo uspelo s trdim delom in kakovostno izvedbo storitev in zaupanih nam nalog, predvsem če bomo sledili naslednjim načelom:

- zagotavljanje celovite ponudbe,
- vedno delo v dobro stranke,
- strokoven razvoj,
- organizacijsko izpopolnjevanje,
- zagotavljanje visoke stopnje kakovosti storitev z upoštevanjem predlogov naših strank,
- ustvarjanje novih delovnih mest,
- povečanje produktivnosti in dobičkonosnosti,
- visoko motiviran in usposobljen kader s primernim vodenjem, kar zagotavlja
- kakovost izvajanja storitev,
- postati vodilno podjetje, ki ponuja rešitve, ki podjetju omogočajo da si na enem
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Article in a journal or newspaper: Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

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

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Članek iz revije ali časopisa: Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

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